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(54) **RECOMBINANT VIRAL VECTORS AND METHODS FOR INDUCING A HETERO SUBTYPIC IMMUNE RESPONSE TO INFLUENZA A VIRUSES**

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*A61K 39/12* (2006.01)  
*A61K 39/00* (2006.01)

(52) **U.S. Cl.**

CPC ..... *A61K 39/285* (2013.01); *A61K 39/12* (2013.01); *A61K 39/145* (2013.01); *A61K 2039/5256* (2013.01); *A61K 2039/70* (2013.01); *C07K 2319/00* (2013.01); *C12N 2710/24143* (2013.01); *C12N 2760/16134* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to recombinant viral vectors and methods of using the recombinant viral vectors to induce an immune response to influenza A viruses. The invention provides recombinant viral vectors based, for example, on the non-replicating modified vaccinia virus Ankara. When administered according to methods of the invention, the recombinant viral vectors are designed to be cross-protective and induce heterosubtypic immunity to influenza A viruses.

**3 Claims, 11 Drawing Sheets**

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## FIGURE 1

**MEKIVLLFAIVSLVKS**DQICIGYHANNSTEQVDTI  
MEKNVTVT~~HA~~QDILEKKHNGKLCGGGGCNTKCQ  
TPMGAINSSMPFHNIHPLTIGBCPKYYVKSNRVLVAT  
GLRNSPQRERRRKKRGLFGAIAGFIEGGWQGMVD  
GWYGYHHSNEQGSGYAADKESTQKAIDGVTNKV  
NSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDG  
FLDVWTYNAELLVLMENERTLDFHDSNVKNLYDK  
VRLQLRDNAKELGNGCFFYHKCDNECMESVRNG  
TYDYPQYSEEARLKREEISGVKLESIGIYQILSIYST  
VASSLALAIMVAGLSLWMCSNGSLQCRICI

## FIGURE 2

ATGGAGAAAATAGTGCTCTTTGCAATAGTCAGTCT  
TGTAAAAAGTGTACAGATTGCATTGGTTACCATGCAA  
ACAACACTCGACAGAGCAGGTTGACACAATAATGGAAA  
AGAACGTTACTGTTACACATGCCAAGACATACTGGA  
AAAGAAACACAACGGGAAGCTCTGCGGAGGAGGAGG  
ATGCAACACCAAGTGTCAAACCTCCAATGGGGCGATA  
AACTCTAGCATGCCATTCCACAATATAACACCCTCTCAC  
CATTGGGAATGCCCAAATATGTGAAATCAAACAGA  
TTAGTCCTTGCAGTGGCTCAGAAATAGCCCTCAAA  
GAGAGAGAAGAAGAAAAAGAGAGGATTATTGGAG  
CTATAGCAGGTTTATAGAGGGAGGATGGCAGGGAAT  
GGTAGATGGTGGTATGGGTACCAACCAGCAATGAG  
CAGGGGAGTGGGTACGCTGCAGACAAAGAATCCACTC  
AAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTC  
GATCATTGACAAAATGAACACTCAGTTGAGGCCGTT  
GGAAGGGAATTAAACAACCTAGAAAGGAGAATAGAG  
AATTAAACAAGAAGATGGAAGACGGGTTCTAGATG  
TCTGGACTTATAATGCTGAACCTCTGGTCTCATGGAA  
AATGAGAGAACTCTAGACTTCTAGACTCAAATGTCA  
AGAACCTTACGACAAGGTCCGACTACAGCTAGGGA  
TAATGCAAAGGAGCTGGTAACGGTTTCGAGTTC  
TATCATAAAATGTGATAATGAATGTATGGAAAGTGTAA  
GAAATGGAACGTATGACTACCCCGAGTATTAGAAGA  
AGCGAGACTAAAAAGAGAGGAAATAAGTGGAGTAAA  
ATTGGAATCAATAGGAATTACCAAATACTGTCAATT  
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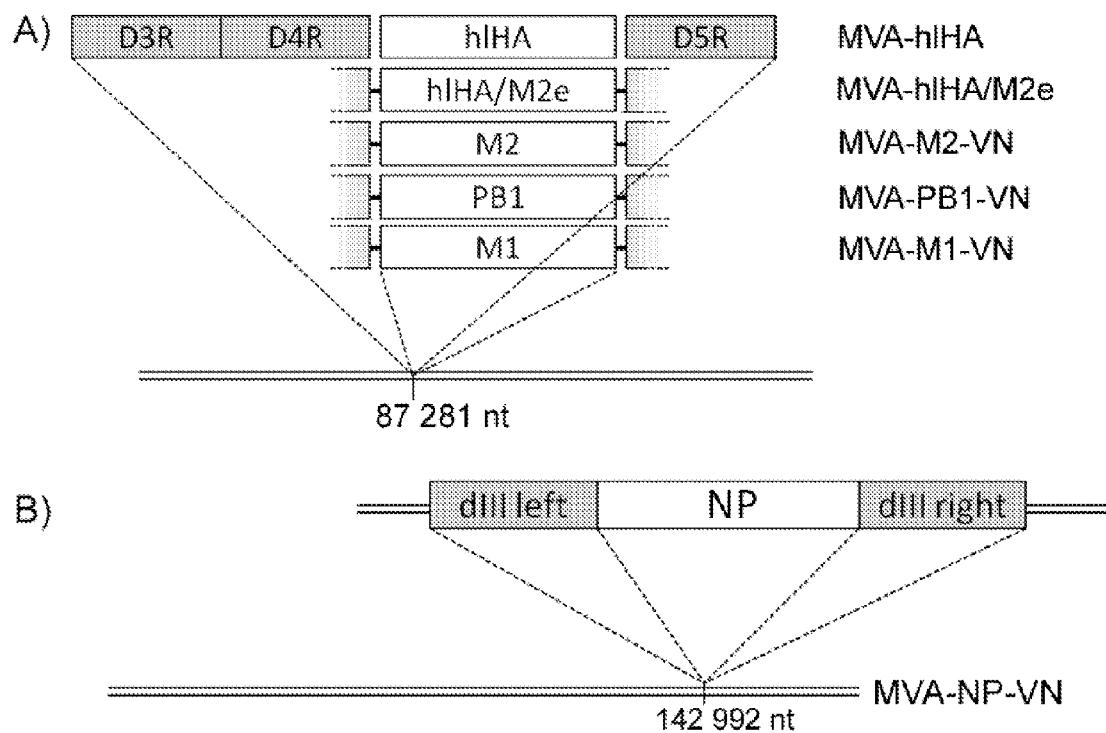
## FIGURE 3

MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIME  
KNVTVTTHAQDILEKKHNGKLCGGGSILTEVETPTRN  
EWECRCSDSSDGSAGSASLLTEVETPIRNEWGCRC  
NDSSDGSASASLLTEVETPTRNGWECKCSDSSDG  
SAGSASLLTEVETPIRKWECNCSDSSDGGGGCNTK  
CQTPMGAINSSMPFHNIHPLTIGECPKYVKSNRLVLAT  
GLRNSPQRERRKKRGLFGAIAGFIEGGWQGMVDG  
WYGYHHSNEQGSGYAADKESTQKAIDGVNKVN  
SIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGF  
LDVWTYNAELLVLMENERTLDFHDSNVKNLYDK  
VRLQLRDNAKELGNGCFEFYHKCDNECMESVRNG  
TYDYPQYSEEARLKREEISGVKLESIGIYQILSIYST  
VASSLALAIMVAGLSLWMCSNGSLQCRICI

## FIGURE 4

ATGGAGAAAATAGTGCTTCTTTGCAATAGTCAGTCTGTTA  
AAAGTGATCAGATTGCATTGGTACCATGCAAACAACACTCGA  
CAGAGCAGGTTGACACAATAATGGAAAAGAACGTTACTGTT  
ACACATGCCAAGACATACTGGAAAAGAAACACAACGGGAA  
GCTCTGCGGAGGAGGAAGTCTTCTAACCGAGGTGAAACGCC  
TACCAGAAACGAATGGGAGTGCAGATGCAGCGATTCAAGTG  
ATGGAAGTGCAGGATCAGCGAGTCTTCTAACCGAGGTGAA  
ACGCCTATCAGAAACGAATGGGGGTGCAGATGCAACGATT  
AAGTGATGGAAGTGCAGGATCAGCGAGTCTTCTAACCGAGGT  
CGAAACGCCTACCAGAAACGGATGGGAGTGCAAATGCAGCG  
ATTCAAGTGATGGAAGTGCAGGATCAGCGAGTCTTCTAACCG  
AGGTGAAACGCCTATCAGAAAAGGATGGGAGTGCAAATGC  
AGCGATTCAAGTGATGGAGGAGGATGCAACACCAAGTGTCA  
AACTCCAATGGGGCGATAAACTCTAGCATGCCATTCCACAA  
TATACACCCTCTCACCATTGGGAATGCCCAAATATGTGAA  
ATCAAACAGATTAGTCTTGCAGTGGCTCAGAAATAGCCC  
TCAAAGAGAGAGAAGAAGAAAAAGAGAGGATTATTGGAG  
CTATAGCAGGTTTATAGAGGGAGGATGGCAGGGAAATGGTA  
GATGGTTGGTATGGTACCAACCATAGCAATGAGCAGGGAG  
TGGTACGCTGCAGACAAAGAATCCACTCAAAAGGCAATAG  
ATGGAGTCACCAATAAGGTCAACTCGATCATTGACAAATGA  
ACACTCAGTTGAGGCCGTTGGAAGGAAATTAAACAAGAAGATGG  
AAAGGAGAATAGAGAATTAAACAAGAAGATGGAAAGACGGG  
TTCCTAGATGTCTGGACTTATAATGCTGAACCTCTGGTTCTCA  
TGGAAAATGAGAGAACTCTAGACTTCTAGACTCAAATGTCA  
AGAACCTTACGACAAGGTCCGACTACAGCTTAGGGATAATG  
CAAAGGAGCTGGTAACGGTGTTCGAGTTCTATCATAAAT  
GTGATAATGAATGTATGGAAAGTGTAAAGAAATGGAACGTAT  
GAECTACCCGCAGTATTCAAGAAGAAGCGAGACTAAAAAGAGA  
GGAAATAAGTGGAGTAAAATTGGAATCAATAGGAATTAC  
AAATACTGTCAATTATTCTACAGTGGCGAGTCCCTAGCACT  
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TGGATCGTTACAATGCAGAATTGCATTAA

FIGURE 5



## FIGURE 6

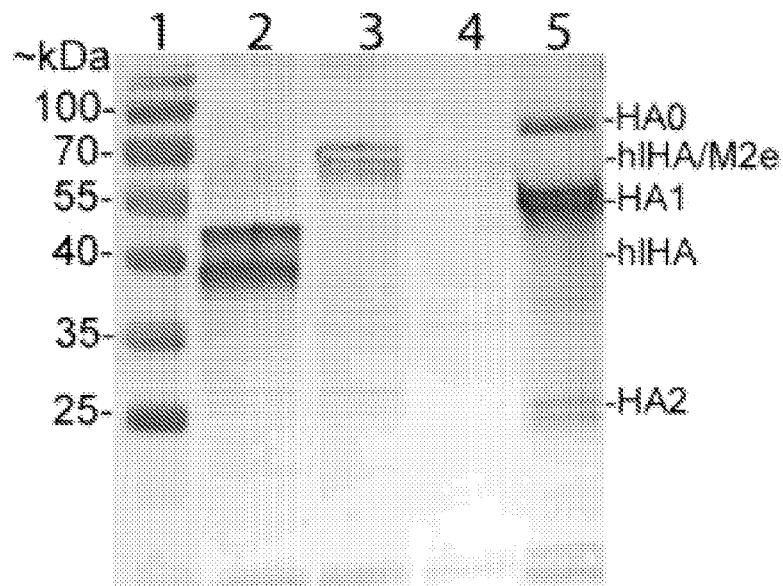
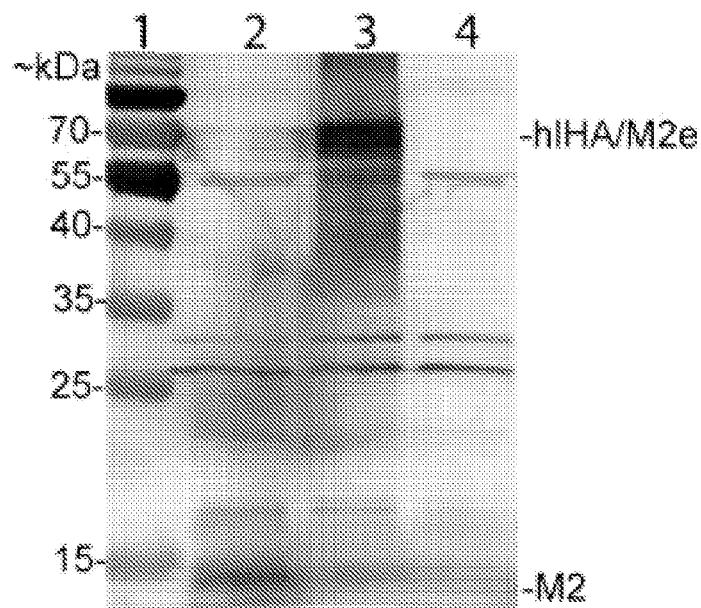
**A****B**

FIGURE 7

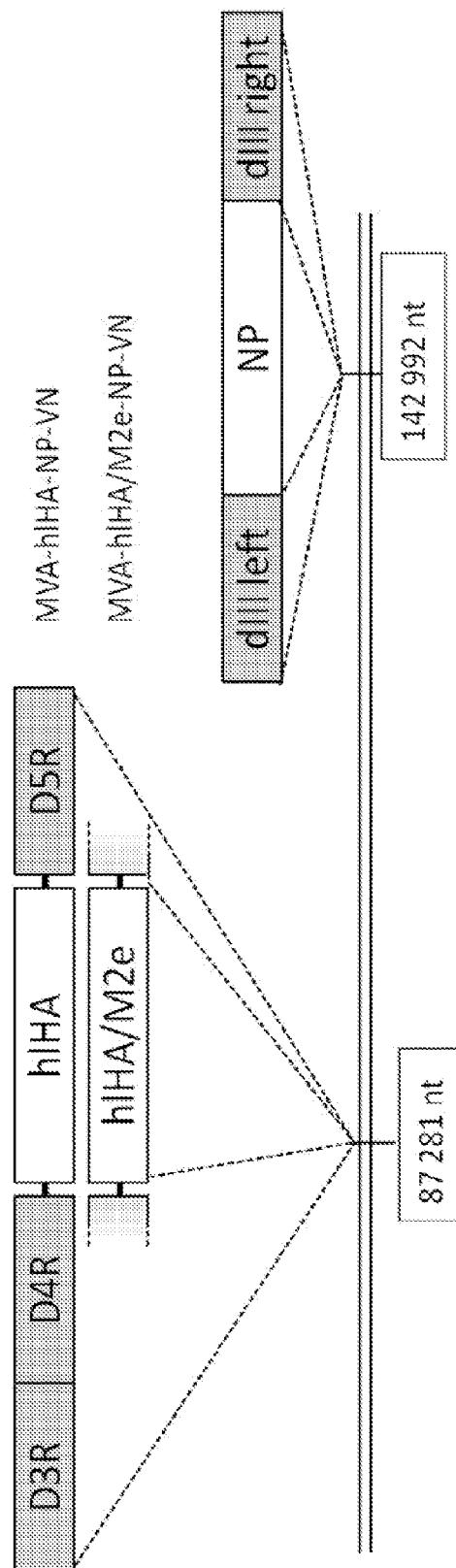


FIGURE 8

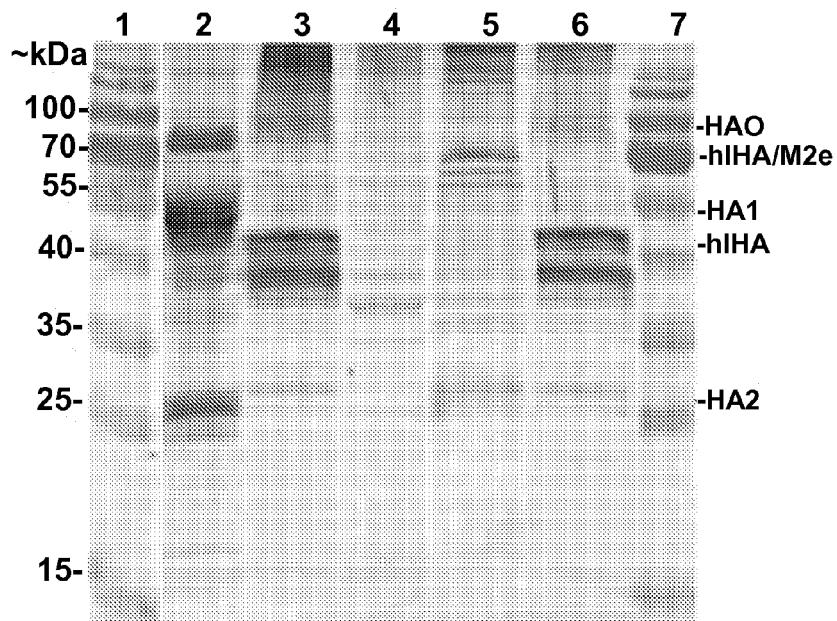
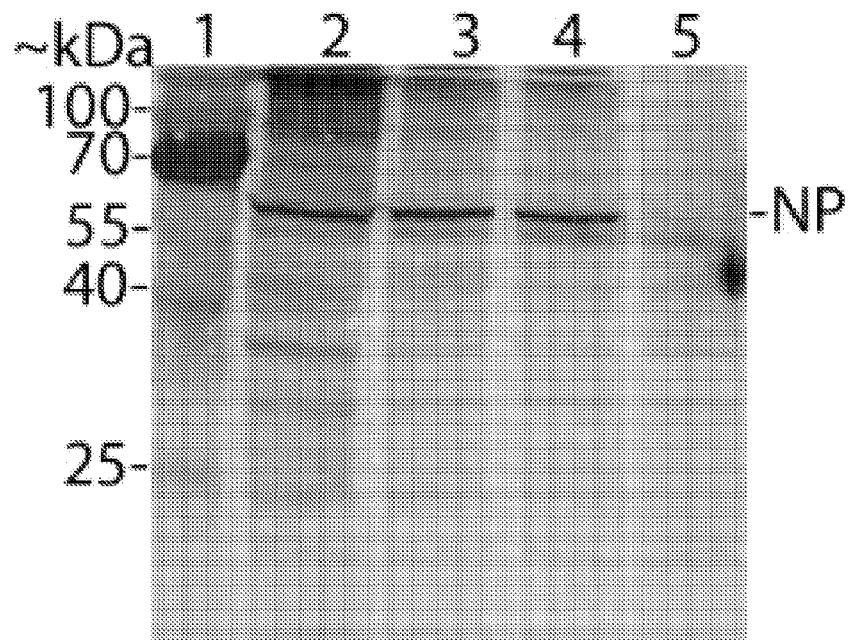
**A****B**

FIGURE 9

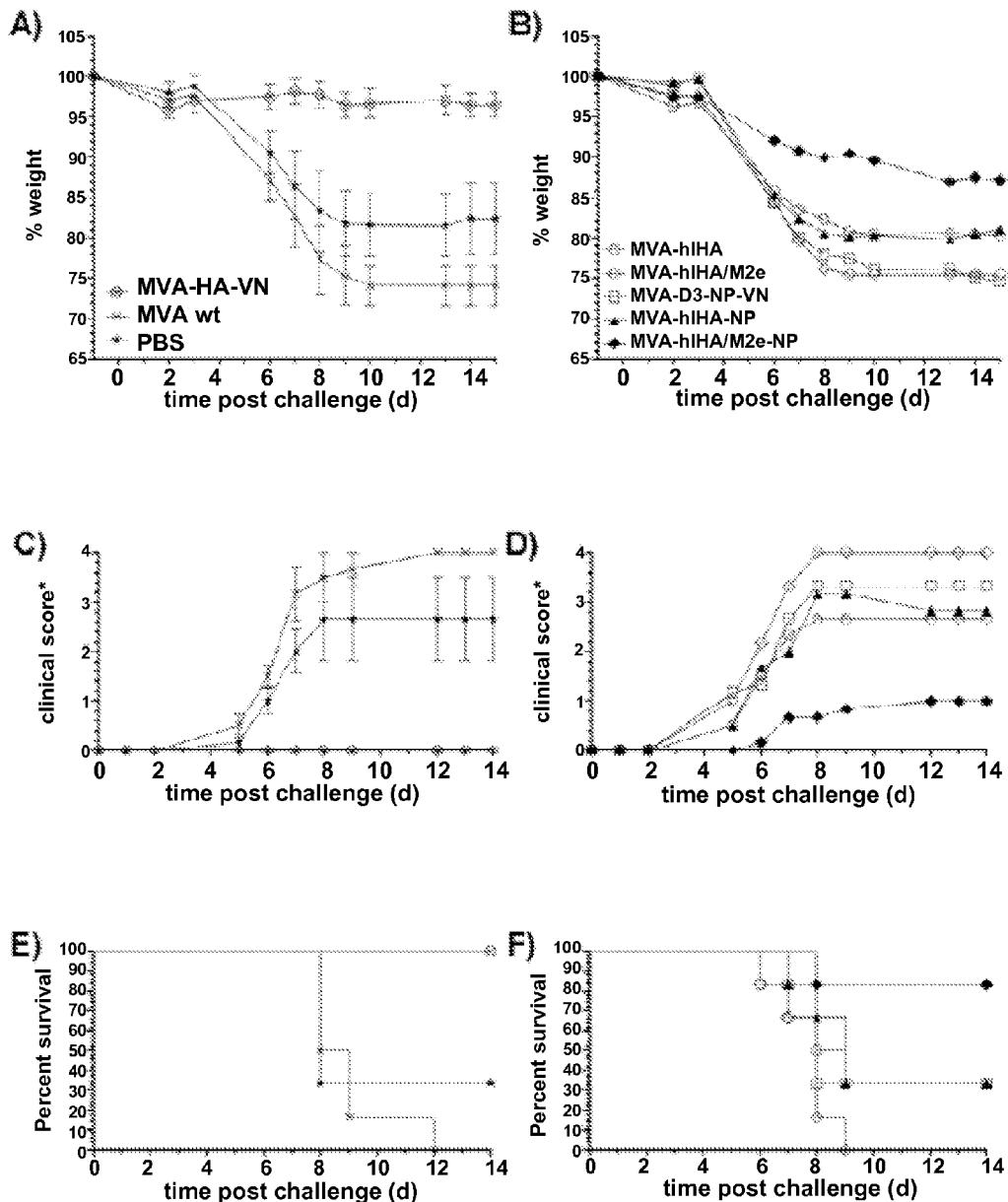


FIGURE 10

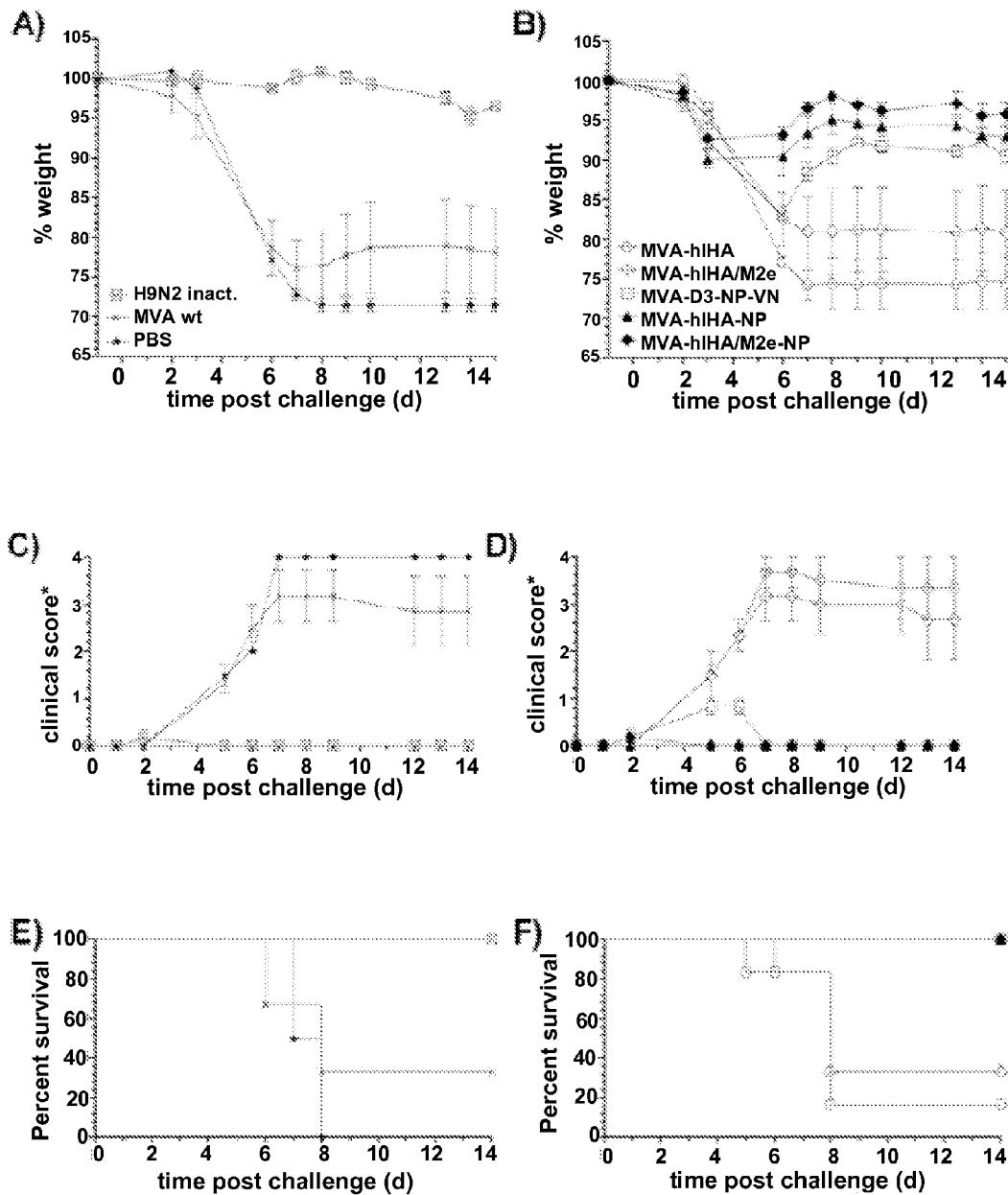
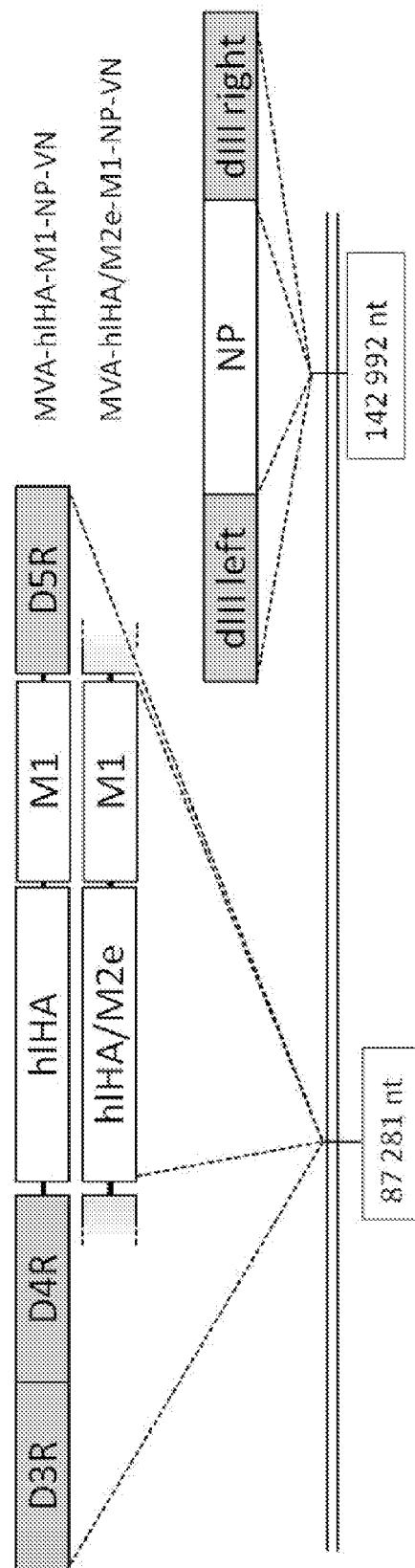


FIGURE 11



## 1

**RECOMBINANT VIRAL VECTORS AND  
METHODS FOR INDUCING A  
HETEROSUBTYPIC IMMUNE RESPONSE  
TO INFLUENZA A VIRUSES**

**FIELD OF THE INVENTION**

The present invention relates to recombinant viral vectors and methods of using the recombinant viral vectors to induce an immune response to influenza A viruses. The invention provides recombinant viral vectors based, for example, on the non-replicating modified vaccinia virus Ankara. When administered according to methods of the invention, the recombinant viral vectors are designed to be cross-protective and induce heterosubtypic immunity to influenza A viruses.

**BACKGROUND OF THE INVENTION**

Human influenza or “the flu” is a respiratory disease that is caused by influenza A and B viruses. Epidemics of influenza cause significant illness and death worldwide each year, and vaccination is the most straightforward strategy to prevent infection and disease. Traditional influenza vaccines expose the recipient to influenza virus proteins causing the recipient to mount an immune response to the proteins. Proteins (or polypeptides) used in vaccines are commonly called “antigens.” The commonly used seasonal influenza vaccines are based on the major antigen of the viruses, the hemagglutinin (HA). There are numerous influenza A subtypes having different HA antigens. Influenza A subtypes are divided and classified based on the HA and neuraminidase (NA) proteins that are expressed by the viruses. The influenza A subtype nomenclature is based on the HA subtype (of the sixteen different HA genes known in the art) and the NA subtype (of the nine different NA genes known in the art). Exemplary subtypes, include, but are not limited to, H5N1, H1N1 and H3N2. There are also variants of the influenza A subtypes which are referred to as “strains.” For example, the virus A/VietNam/1203/2004 is an influenza A virus, subtype H5N1, with a strain name A/VietNam/1203/2004.

Protection from the seasonal vaccines based on the HA is strain-specific and new strains emerge constantly, so the classical influenza vaccines have to be re-formulated each year in an attempt to match the currently circulating strains. See, Lambert and Fauci 2010. It is therefore highly desirable for next generation vaccines to be cross-protective and induce heterosubtypic immunity, i.e., vaccines against one subtype that protect or partially protect against challenge infection with influenza A of different subtypes.

The current ‘universal vaccines’ (i.e., vaccines designed to elicit heterosubtypic immunity) that are under development are mainly based on the more conserved internal influenza virus genes including the influenza matrix proteins (M1 and M2) (Schotsaert et al. 2009), the nucleoprotein (NP) and conserved parts of the HA (Bommakanti et al. 2010; Steel et al. 2010). The polymerase proteins PA, PB1 and PB2 also induce substantial T cell responses and may be also relevant targets (Assarsson et al. 2008; Greenbaum et al. 2009; Lee et al. 2008).

Next generation influenza vaccines currently under development include recombinant proteins, synthetic peptides, virus-like particles (VLPs), DNA-based vaccines and viral vector vaccines (Lambert and Fauci, *supra*). The advantage of using live viral vectors is their known property to induce high levels of cellular immunity, in particular CD8 T cells. Among the most promising viral vectors are vaccinia virus-

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based live vaccines (Rimmelzwaan and Sutter 2009) and adenovirus-based vectors (Hoelscher et al. 2006; Hoelscher et al. 2007; Price et al. 2010; Zhou et al. 2010). Single-dose mucosal immunization using an adenovirus construct expressing NP and M2, for instance, provided protection from virulent H5N1, H3N2 and H1N1 viruses (Price et al, *supra*). In a further study (Price et al. 2009), DNA vaccination with nucleoprotein (NP) and matrix 2 (M2) plasmids followed by boosting with antigen-matched recombinant adenovirus (rAd) provided robust protection against virulent H1N1 and H5N1 challenges in mice and ferrets.

Recombinant vaccines based on modified vaccinia virus Ankara (MVA) have been used in many non-clinical and clinical studies. MVA has proven to be exceptionally safe.

No significant side effects have been obtained when MVA was administered to more than 120,000 human patients in the context of the smallpox eradication. Due to a block in virion morphogenesis the highly attenuated vaccinia virus strain fails to productively replicate in human and most other mammalian cells. Nevertheless, the ability to express viral and foreign genes in the early and late stage is retained. These characteristics make MVA a promising live vaccine vector that induces humoral and cellular immune responses and that exhibits a high safety profile.

U.S. Pat. Nos. 6,998,252; 7,015,024; 7,045,136 and 7,045,313 relate to recombinant poxviruses, such as vaccinia.

MVA-based vaccines have been used in clinical studies, for instance, against HIV, tuberculosis, malaria and cancer.

In all of these studies, at least two doses were used. The human dose of an MVA-based vaccine was  $5 \times 10^7$  to  $5 \times 10^8$  PFU as applied in clinical trials (Brookes et al. 2008; Cebere et al. 2006; Tykodi and Thompson 2008;).

MVA has been used recently as a vector in pandemic H5N1 (Kreijtz et al. 2008; Kreijtz et al. PLoS One 2009; Kreijtz et al. Vaccine 2009; Kreijtz et al. J. Infect. Dis. 2009; Kreijtz et al. 2007; Mayrhofer et al., 2009; Poon et al. 2009) and H1N1 (Hessel et al. 2010; Kreijtz et al., J. Infect. Dis. 2009) influenza research. An MVA-based vaccine expressing NP and M1 is currently being tested in an ongoing clinical trial (Berthoud et al. 2011).

Thus, there remains a need in the art for a more broadly protective influenza vaccine.

**DETAILED DESCRIPTION**

The present invention provides recombinant viruses (also referred to as recombinant viral vectors herein) useful for generating a heterosubtypic immune response to influenza A viruses. The recombinant viruses are recombinant vaccinia viruses, such as recombinant MVA or other non-replicating or replicating vaccinia virus known in the art. Non-replicating vaccinia viruses include, but are not limited to, defective vaccinia Lister (dVV), MVA-575 (ECACC V00120707),

MVA-BN (ECACC V00083008), MVA-F6 and MVA-M4 (Antoine et al. 1998). In some embodiments, the recombinant viruses encode a fusion protein (hlHA/M2e) comprising an influenza A hemagglutinin deletion mutant “headless HA” (hlHA) with at least one influenza A M2 external domain (M2e) insert; an hlHA/M2e fusion protein and an influenza A nucleoprotein (NP); or an hlHA and NP. The recombinant viruses of the invention may further encode an influenza A matrix protein 1 (M1) and/or an influenza A polymerase PB1. When administered according to methods of the invention, the recombinant viruses are cross-protective and induce heterosubtypic humoral and cellular immune responses (including CD8 and CD4 T cell responses). The

60 65

recombinant viruses are therefore contemplated to be useful as universal influenza A vaccines in humans.

In some embodiments, the hIHA amino acid sequence encoded by an open reading frame in recombinant viruses of the invention may be, for example, the hIHA amino acid sequence set out in SEQ ID NO: 15 (based on A/VietNam/1203/2004 H5N1 HA NCBI Genbank AAW80717 which is SEQ ID NO: 3). The hIHA of SEQ ID NO: 15 comprises a signal sequence, the HA1 residues 17-58 of SEQ ID NO: 3, a linker peptide of four glycines, the HA1 residues 290-343 of SEQ ID NO: 3 and the HA2 stalk region residues 344-568 of SEQ ID NO: 3.

In some embodiments, the hIHA/M2e fusion protein amino acid sequence encoded by an open reading frame in recombinant viruses of the invention may be, for example, the hIHA/M2e fusion protein amino acid sequence set out in SEQ ID NO: 2. The fusion protein of SEQ ID NO: 2 comprises a signal sequence, the HA1 residues 17-58 of SEQ ID NO: 3, a linker peptide of three glycines (SEQ ID NO: 4), the M2e of H5N1 (SEQ ID NO: 5 based on A/VietNam/1203/2004 H5N1 NCBI Genbank ABP35634), a six-amino acid linker GSAGSA (SEQ ID NO: 9), the M2e of H1N1 (equivalent to H2N2 and H3N2) (SEQ ID NO: 6 based on A/New York/3315/2009 H1N1 NCBI Genbank ACZ05592), a six-amino acid linker GSAGSA (SEQ ID NO: 9), the M2e of H9N2 (SEQ ID NO: 7 based on A/chicken/Korea/SH0913/2009 H9N2 NCBI Genbank ADQ43641), a six-amino acid linker GSAGSA (SEQ ID NO: 9), the M2e of H7N2 (SEQ ID NO: 8 based on A/New York/107/2003 H7N2 NCBI Genbank ACC55276), a linker peptide of three glycines (SEQ ID NO: 4), the HA1 residues 290-343 of SEQ ID NO: 3 and the HA2 region residues 344-568 of SEQ ID NO: 3.

In some embodiments, the hIHA/M2e fusion protein may comprise one, two, three or four of the M2e polypeptides of SEQ ID NOs: 5, 6, 7 and 8. The hIHA/M2e fusion protein may comprise an influenza A M2e polypeptide other than an M2e polypeptide of SEQ ID NOs: 5, 6, 7, and 8.

In some embodiments, the NP amino acid sequence encoded by an open reading frame in recombinant viruses of the invention may be, for example, the NP amino acid sequence set out in SEQ ID NO: 13 (based on A/VietNam/1203/2004 H5N1 NP NCBI Genbank AAW80720). In some embodiments, the M1 amino acid sequence encoded by an open reading frame in recombinant viruses of the invention may be, for example, the M1 amino acid sequence set out in SEQ ID NO: 11 (based on A/VietNam/1203/2004 H5N1 M1 Genbank AAW80726). In some embodiments, the PB1 amino acid sequence encoded by an open reading frame in recombinant viruses of the invention may be, for example, the PB1 amino acid sequence set out in SEQ ID NO: 17 (based on A/VietNam/1203/2004 H5N1 PB1 Genbank AAW80711).

The invention contemplates that polypeptides encoded by an open reading frame in a recombinant virus may vary in sequence from SEQ ID NO: 2, 5, 6, 7, 8, 11, 13, 15 and/or 17 if the polypeptides retain the ability to induce a protective

immune response when the recombinant virus is administered to an individual. In these embodiments, the polypeptide may be about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 95%, about 97%, about 98% or about 99% identical to SEQ ID NO: 2, 5, 6, 7, 8, 11, 13, 15 and/or 17.

In other embodiments, hIHA/M2e fusion proteins, hIHA polypeptides and NP polypeptides encoded by recombinant viruses of the invention may be based on the same or different influenza A subtypes including, but not limited to, any combination of H1 to H16 and N1 to N9 (including H1N1, H2N1, H3N1, H4N1, H5N1, H6N1, H7N1, H8N1, H9N1, H10N1, H11N1, H12N1, H13N1, H14N1, H15N1, H16N1; H1N2, H2N2, H3N2, H4N2, H5N2, H6N2, H7N2, H8N2, H9N2, H10N2, H11N2, H12N2, H13N2, H14N2, H15N2, H16N2; H1N3, H2N3, H3N3, H4N3, H5N3, H6N3, H7N3, H8N3, H9N3, H10N3, H11N3, H12N3, H13N3, H14N3, H15N3, H16N3; H1N4, H2N4, H3N4, H4N4, H5N4, H6N4, H7N4, H8N4, H9N4, H10N4, H11N4, H12N4, H13N4, H14N4, H15N4, H16N4; H1N5, H2N5, H3N5, H4N5, H5N5, H6N5, H7N5, H8N5, H9N5, H10N5, H11N5, H12N5, H13N5, H14N5, H15N5, H16N5; H1N6, H2N6, H3N6, H4N6, H5N6, H6N6, H7N6, H8N6, H9N6, H10N6, H11N6, H12N6, H13N6, H14N6, H15N6, H16N6; H1N7, H2N7, H3N7, H4N7, H5N7, H6N7, H7N7, H8N7, H9N7, H10N7, H11N7, H12N7, H13N7, H14N7, H15N7, H16N7; H1N8, H2N8, H3N8, H4N8, H5N8, H6N8, H7N8, H8N8, H9N8, H10N8, H11N8, H12N8, H13N8, H14N8, H15N8, H16N8; H1N9, H2N9, H3N9, H4N9, H5N9, H6N9, H7N9, H8N9, H9N9, H10N9, H11N9, H12N9, H13N9, H14N9, H15N9, and H16N9). In some embodiments the influenza A subtype is a pandemic influenza A. Exemplary pandemic influenza subtypes include, but are not limited to, H1N1, H2N2, H3N2 and H5N1.

A list of identified Influenza A strains, including influenza A H1N1 strains, is available from the World Health Organization (WHO) and United States Centers for Disease Control (CDC) databases of Influenza A subtypes. The National Center for Biotechnology Information (NCBI) database maintained by the United States National Library of Medicine also maintains an updated database describing the length and sequence of HA, M2, NP, M1 and PB1 genes of viruses of influenza A species. Strains listed by these organizations and strains described in other commercial and academic databases, or in literature publications and known in the art, are contemplated for use in the invention. It is also contemplated that additional influenza A strains hereafter identified and isolated are also useful in the invention as sources of influenza A protein sequences. Accordingly, any strain specifically exemplified in the specification and those known or after discovered in the art are amenable to the recombinant vaccinia virus, pharmaceutical compositions, and methods of the invention. Exemplary strains include, but are not limited to, the strains in Table 1 below. The table also lists exemplary genes and associated database accession numbers of those strains.

TABLE 1

Virus subtype	Inserted Influenza gene	Virus strain	NCBI gene acc no.	NCBI amino acid acc no.
H5N1	HA	A/Viet Nam/1203/2004	AY818135	AAW80717
H5N1	NP	A/Viet Nam/1203/2004	AY818138	AAW80720
H5N1	M1	A/Viet Nam/1203/2004	AY818144	AAW80726
H5N1	PB1	A/Viet Nam/1203/2004	AY818129	AAW80711

TABLE 1-continued

Virus subtype	Inserted Influenza gene	Virus strain	NCBI gene acc no.	NCBI amino acid acc no.
H5N1	M2	A/Viet Nam/1203/2004	EF541453	ABP35634
H1N1 sw	M2	A/California/07/09	FJ969537	ACP44185
H1N1	M2	A/New York/3315/2009	CY050765	ACZ05592
H2N2	M2	A/Korea/426/68	NC_007377	YP_308853
H3N2	M2	A/New York/392/2004	NC_007367	YP_308840
H9N2	M2	A/chicken/Korea/SH0913/2009	HQ221654	ADQ43641
H7N2	M2	A/New York/107/2003	EU587373	ACC55276
H7N3	M2	A/chicken/Pakistan/34668/1995	CY035834	ACJ03948

In recombinant viruses of the invention, open reading frames encoding hIHA/M2e, hIHA, NP, M1 and/or PB1 may be codon-optimized for expression in human cells. In these embodiments, one or more (or all) of the naturally occurring codons in an open reading frame have been replaced in the codon-optimized open reading frame with codons frequently used in genes in human cells (sometimes referred to as preferred codons). Codons may be optimized to avoid repeat sequences to stabilize an open reading frame in the rMVA and/or to avoid unwanted transcription stop signals. Codon-optimization, in general, has been used in the field of recombinant gene expression to enhance expression of polypeptides in cells.

Gene cassettes encoding hIHA/M2e, hIHA, NP, M1 and PB1 in recombinant viruses of the invention include an open reading frame under the control of (i.e., operatively linked to) a promoter that functions (i.e., directs transcription of the open reading frame) in the recombinant vaccinia viruses. In exemplary embodiments, expression from gene cassettes is under the control of the strong early/late vaccinia virus mH5 promoter (SEQ ID NO: 18) or the synthetic early/late selP promoter (SEQ ID NO: 19) (Chakrabarti et al. 1997). In the gene cassettes of the invention the open reading frame is also operatively linked to a transcription stop signal such as a vaccinia virus early transcription stop signal.

In one aspect, the invention provides recombinant vaccinia virus comprising a gene cassette encoding an influenza A hIHA/M2e fusion protein. In some embodiments, the recombinant vaccinia virus is a recombinant MVA comprising a gene cassette expressing the hIHA/M2e fusion protein set out in SEQ ID NO: 2. In some embodiments, the recombinant vaccinia virus further comprises a gene cassette expressing the M1 protein (for example, the M1 set out in SEQ ID NO: 11) and/or a gene cassette expressing the PB1 protein (for example, the PB1 protein set out in SEQ ID NO: 17).

In another aspect, the invention provides recombinant vaccinia virus comprising a first gene cassette encoding an influenza A hIHA/M2e fusion protein, and a second gene cassette encoding an influenza NP. In some embodiments, the recombinant vaccinia virus is a recombinant MVA comprising a first gene cassette expressing the hIHA/M2e fusion protein set out in SEQ ID NO: 2 and a second gene cassette expressing the NP set out in SEQ ID NO: 13. In some embodiments, the recombinant vaccinia virus further comprises a gene cassette expressing the M1 protein (for example, the M1 set out in SEQ ID NO: 11) and/or a gene cassette expressing the PB1 protein (for example, the PB1 protein set out in SEQ ID NO: 17).

In yet another aspect, the invention provides recombinant vaccinia virus comprising a first gene cassette encoding an influenza A hIHA and a second gene cassette encoding an influenza NP. In some embodiments, the recombinant vac-

cinia virus is a recombinant MVA comprising a first gene cassette expressing the hIHA set out in SEQ ID NO: 15 and a second gene cassette expressing the NP set out in SEQ ID NO: 13. In some embodiments, the recombinant vaccinia virus further comprises a gene cassette expressing the M1 protein (for example, the M1 set out in SEQ ID NO: 11) and/or a gene cassette expressing the PB1 protein (for example, the PB1 protein set out in SEQ ID NO: 17).

In recombinant vaccinia viruses of the invention, the gene cassettes may be inserted in non-essential regions of the vaccinia virus genome, such as the deletion I region, the deletion II region, the deletion III region, the deletion IV region, the thymidine kinase locus, the D4R/5R intergenic region, or the HA locus. In exemplified embodiments of recombinant MVA, the insertion of the hIHA/M2e and hIHA gene cassettes is in the D4R/5R intergenic region and the insertion of the NP gene cassette is in the deletion III region. The recombinant MVA is derived from an MVA free of bovine spongiform encephalopathy (BSE) such as MVA74 LVD6 obtained from the National Institutes of Health.

The recombinant viruses of the invention may be formulated as pharmaceutical compositions according to methods known in the art. In some embodiments, the recombinant viruses are formulated as described in International Publication No. WO 2010/056991.

The invention provides methods of inducing a heterosubtypic influenza A immune response in an individual comprising administering compositions of recombinant vaccinia virus of the invention to the individual. In the methods, the composition may be administered as a single dose, a double dose or multiple doses. The administration route in humans may be inhalation, intranasally, orally, and parenterally. Examples of parenteral routes of administration include intradermal, intramuscular, intravenous, intraperitoneal and subcutaneous administration. The range of the human immunization dose may be about 10<sup>6</sup> to about 10<sup>9</sup> PFU. The methods of the invention induce humoral and cellular immune responses in the individual. Moreover, in embodiments of the invention the methods induce a protective immune response in the individual. The protective immune response may be where the individual exhibits no symptoms of infection, a reduction in symptoms, a reduction in virus titer in tissues or nasal secretions, and/or complete protection against infection by influenza virus.

The invention also provides kits for administering recombinant vaccinia virus of the invention packaged in a manner which facilitates their use to practice methods of the invention. In one embodiment, such a kit includes a recombinant virus or composition described herein, packaged in a container such as a sealed bottle or vessel, with a label affixed to the container or included in the package that describes use of the compound or composition in practicing the method. Preferably, the recombinant virus or composition is pack-

aged in a unit dosage form. The kit may further include a device suitable for administration according to a specific route of administration or for practicing a screening assay. Preferably, the kit contains a label that describes use of the recombinant vaccinia virus. In some embodiments, the kit comprises instructions for administration to a human subject.

Also provided are methods of producing a recombinant vaccinia virus expressing a gene cassette of the invention. As illustrated with MVA, the methods comprise the steps of: a) infecting primary chicken embryo cells or a suitable permanent cell line (e.g., avian) with MVA, b) transfecting the infected cells with a plasmid comprising the gene cassette and comprising DNA flanking the gene cassette that is homologous to a non-essential region of the MVA genome, c) growing the cells to allow the plasmid to recombine with the MVA genome during replication of the MVA in chicken cells thereby inserting the gene cassette into the MVA genome in the non-essential region, and d) obtaining the recombinant MVA produced. Exemplary chicken embryo cells are described in U.S. Pat. No. 5,391,491. (Slavik et al. 1983) Other avian cells (e.g., DF-1) are also contemplated. In the methods, the non-essential MVA region is the deletion I region, the deletion II region (Meyer et al. 1991), the deletion III region (Antoine et al. 1996), the deletion IV region (Meyer et al., supra; Antoine et al. 1998) the thymidine kinase locus (Mackett et al. 1982), the D4R/5R intergenic region (Holzer et al. 1998), or the HA locus (Antoine et al. supra). In one exemplified embodiment, the insertion is in the deletion III region. In another exemplified embodiment, the insertion is in the D4R/5R intergenic region. If two gene cassettes are to be inserted, the two are inserted in different non-essential regions. Gene cassettes may additionally be inserted into any other suitable genomic region or intergenic regions.

Other vertebrate cell lines are useful for culture and growth of vaccinia virus of the invention. Exemplary vertebrate cells useful to culture vaccinia virus of the invention include, but are not limited to, MRC-5, MRC-9, CV-1 (African Green monkey), HEK (human embryonic kidney), PerC6 (human retinoblast), BHK-21 cells (baby hamster kidney), BSC (monkey kidney cell), LLC-MK2 (monkey kidney) and permanent avian cell lines such as DF-1.

Vero cells are an accepted cell line for production of viral vaccines according to the World Health Organization. In some embodiments, recombinant replicating vaccinia virus of the invention are produced in Vero cells.

Additional aspects and details of the invention will be apparent from the following examples, which are intended to be illustrative rather than limiting.

## FIGURES

FIG. 1 shows the amino acid sequence (SEQ ID NO: 15) of the headless HA protein encoded by recombinant MVA (rMVA) of the invention. The protein contains a signal sequence (grey), HA1 residues (red), a linker peptide of four glycines (black), HA1 residues (red), and the HA2 stalk region (black). Cysteines 58 and 63 and the polybasic cleavage site (amino acids 112-119) are underlined.

FIG. 2 shows the nucleotide sequence (SEQ ID NO: 14) of the headless HA protein encoded by rMVA of the invention.

FIG. 3 shows the amino acid sequence (SEQ ID NO: 2) of headless HA/M2e fusion protein. The designed protein contains a signal sequence (grey), HA1 residues (red), a linker peptide of three glycines (black), the M2e of H5N1

(blue), the six amino acid linker GSAGSA (black), the M2e of H1N1 (equivalent to H2N2, H3N2; green), the six amino acid linker GSAGSA, the M2e of H9N2 (orange), the six amino acid linker GSAGSA, the M2e of H7N2 (pink), a linker peptide of three glycines (black), HA1 residues (red) and the HA2 stalk region (black). The polybasic cleavage site (amino acids 224-231) is underlined.

FIG. 4 shows the nucleotide sequence (SEQ ID NO: 1) of the headless HA/M2e fusion protein encoded by rMVA of the invention.

FIG. 5 shows single-insert rMVAs containing influenza genes. FIG. 5A indicates the hlHA, hlHA/M2e, M2, PB1, or M1 gene cassettes that are located in the recombinant MVA D4R/D5R intergenic locus, at the position corresponding to nucleotide 87,281 of wild type MVA (Antoine et al, supra). FIG. 5B indicates the NP gene cassette is located in the del III locus at the position corresponding to nucleotide 142,992 of wild type MVA.

FIG. 6 shows a Western Blot of chicken cell lysates tested for influenza virus antigens. A) Expression of headless HA and the headless HA/M2e fusion protein using a detection antibody directed against HA. Lane 1, protein ladder, size in kDa; lane 2, MVA-hlHA; lane 3, MVA-hlHA/M2e; lane 4, MVA wt (negative control); and lane 5, MVA-HA-VN (positive control). B) Expression of the headless HA/M2e fusion protein using a detection antibody directed against M2. Lane 1, protein ladder, size in kDa; lane 2, MVA-M2-VN; lane 3, MVA-hlHA/M2e; and lane 4, MVA wt (negative control). The recombinant MVA-M2-VN expresses the M2 protein (weak band below 15 kDa). The anti-M2-antibody binds a peptide at the N-terminus of the M2 protein; thus the expression of the hlHA/M2e fusion protein is also detectable at around 70 kDa (lane 3).

FIG. 7 shows double-insert rMVAs containing influenza genes. The hlHA or hlHA/M2e gene cassette is located in the D4R/D5R intergenic locus, at the position corresponding to nucleotide 87,281 of wild type MVA. The NP gene cassette is located in the del III locus at the position corresponding to nucleotide 142,992 of the wild type MVA.

FIG. 8 shows a Western Blot of chicken cell lysates tested for influenza virus antigens. A) Expression of headless HA and the headless HA/M2e fusion protein using a detection antibody directed against HA. Lanes 1 and 7, protein ladder, size in kDa; lane 2, MVA-HA-VN (positive control); lane 3, MVA-hlHA; lane 4, MVA wt (negative control); lane 5, MVA-hlHA/M2e-NP; and lane 6, MVA-hlHA-NP. The hlHA/M2e fusion protein expressed by MVA-hlHA/M2e is visible at around 70 kDa (lane 5). The lower bands at around 40 kDa represent the hlHA expressed by MVA-hlHA-NP and MVA-hlHA. The control construct (MVA-HA-VN), expressing the full length HA protein express the HA0 (band around 80 kDa), the HA1 (band around 55 kDa, and the HA2 (band around 25 kDa). The expression of the HA2 protein is also visible in lanes 3, 5 and 6 as the hlHA and hlHA/M2e proteins also contain the polybasic cleavage site. The specific HA bands are absent in the negative control (lane 4). B) NP expression detected with an NP-specific antibody. Lane 1, protein ladder, size in kDa; lane 2, MVA-D3-NP-VN; lane 3, MVA-hlHA-NP; lane 4, MVA-hlHA/M2e-NP; and lane 5, MVA wt (negative control).

FIG. 9 shows monitoring of weight (A, B), clinical symptoms (C, D) and survival (E, F) after vaccination with recombinant MVAs and challenge with H5N1. As controls, mice were vaccinated with MVA-HA-VN, expressing the full-length HA of A/Vietnam/1203/2004, wt MVA or were treated with PBS (panels A, C, E). Mice were vaccinated with the single recombinant MVA-hlHA, MVA-hlHA/M2e,

MVA-NP-VN or the double recombinants MVA-hlHA-NP and MVA-hlHA/M2e-NP (panels B, D, F). After challenge with wild-type H5N1, mice were monitored for 14 days.

FIG. 10 shows monitoring of weight (A, B), clinical symptoms (C, D) and survival (E, F) after vaccination with recombinant MVAs and challenge with H9N2 virus. As controls, mice were vaccinated with the whole virus preparation of H9N2, wt MVA or were treated with PBS (panels A, C, E). Mice were vaccinated with the single recombinant MVA-hlHA, MVA-hlHA/M2e, MVA-NP-VN or double recombinant MVA-hlHA-NP and MVA-hlHA/M2e-NP (panels B, D, F). After challenge with virulent mouse-adapted H9N2 influenza virus, mice were monitored for 14 days.

FIG. 11 shows triple-insert rMVAs containing influenza genes. The hlHA or hlHA/M2e and M1 gene cassettes will be located in the D4R/D5R intergenic locus, at the position 87,281 nt of the wt MVA sequence. The NP gene cassette will be located in the del III locus at the position 142,992 nt of the wt MVA sequence.

#### EXAMPLES

The present invention is illustrated by the following examples wherein Example 1 describes the choice and design of influenza A antigens in exemplary recombinant MVA of the invention, Example 2 details the production of single-insert recombinant MVAs, Example 3 describes animal experiments with the single-insert MVAs, Example 4 details the production of double-insert recombinant MVAs, Example 5 describes animal experiments with the double-insert MVAs, Example 6 details the production of triple-insert recombinant MVAs and Example 7 describes animal experiments with the triple-insert MVAs.

#### Example 1

##### Choice and Design of Influenza A Antigens

Influenza headless HA, a headless HA/M2e fusion protein, NP, M1, M2 and PB1 were the influenza A antigens chosen to be encoded by exemplary recombinant MVA of the invention.

Monoclonal antibodies against the HA stalk domain, the HA2 region, are broadly cross-reactive and neutralize several subtypes of viruses (Ekiert et al. 2009; Kashyap et al. 2008; Okuno et al. 1993; Sanchez-Fauquier et al. 1987; Sui et al. 2009; Throsby et al. 2008). The antibodies target the HA2 region of the molecule and presumably act by preventing the conformational change of HA at low pH, thus presumably blocking fusion of viral and host membranes during influenza infection. However, the production of soluble, native (neutral pH-like) HA2 immunogen has proven to be difficult, owing to the metastable nature of HA (Chen et al. 1995). To induce an immune response against the neutral pH conformation, a headless HA was chosen as an antigen. The headless HA consists of two HA1 regions that interact with an HA2 subunit, stabilizing the neutral pH conformation (Bommakanti et al., *supra*; Steel et al., *supra*).

The extracellular domain of the M2 protein (M2e, 23AS) is highly conserved across influenza A virus subtypes. In animals, M2e specific antibodies reduce the severity of infection with a wide range of influenza A virus strains (Fan et al. 2004; Neirynck et al. 1999). Many groups have reported M2e-based vaccine candidates in different forms (De Filette et al. 2008; Denis et al. 2008; Eliasson et al. 2008; Fan et al., *supra*; Neirynck et al., *supra*). Recently,

Zhao et al. reported that a tetra-branched multiple antigenic peptide vaccine based on H5N1 M2e induced strong immune responses and cross protection against different H5N1 clades and even heterosubtypic protection from 2009 H1N1 (Zhao et al. 2010b; Zhao et al. 2010a).

Vaccination using vectors expressing the conserved influenza NP, or a combination of NP and matrix protein has been studied in animal models and various degrees of protection against both homologous and heterologous viruses have been demonstrated (Price et al., *supra*; Ulmer et al. 1993). NP elicit a robust CD8<sup>+</sup> T cell response in mice and in humans (McMichael et al. 1986; Yewdell et al., 1985) that, as epidemiological studies suggest, may contribute to resistance against severe disease following influenza A virus infection (Epstein 2006).

The headless HA included in rMVA of the invention is a new headless HA (hlHA) based on the VN/1203 influenza strain. The hlHA contains a polybasic cleavage site which is cleaved during expression from the rMVA exposing the fusion peptide for the immune system. The amino acid sequence of the hlHA is set out in FIG. 1 and in SEQ ID NO: 15. The nucleotide sequence of the MVA insert is set out FIG. 2 and SEQ ID NO: 14.

The amino acid sequence of the headless HA/M2e fusion protein included in rMVA of the invention is set out in FIG. 3 below and in SEQ ID NO: 2. The nucleotide sequence of the fusion protein is set out in FIG. 4 below and in SEQ ID NO: 1. In the fusion protein, the M2e domains of H5N1, H9N2, H7N2 and H1N1 (equivalent to H2N2, H3N2) form an M2e "head" on the hlHA. The four particular M2e domains were chosen to represent the M2e from seasonal and pandemic strains.

#### Example 2

##### Construction and Characterization of Single-Insert MVA Vectors

The following single-insert, recombinant MVA (rMVA) are utilized in the experiments described herein.

TABLE 2

rMVA	Inserted influenza gene	NCBI gene acc no.
1. MVA-hlHA	headless HA	based on AY818135
2. MVA-hlHA/M2e	headless HA/M2e fusion	based on AY818135
3. MVA-M1-VN	Matrix protein 1	AY818144
4. MVA-M2-VN	Matrix protein 2	EF541453
5. MVA-PB1-VN	Polymerase subunit PB1	AY818129
6. MVA-mNP	Nucleoprotein	AY818138
7. Control MVA-HA-VN	Hemagglutinin	AY818135
8. Control MVA-wt	No insert	—
9. Control PBS	No insert	—

For construction of single-insert rMVA vectors expressing hlHA, the hlHA/M2e fusion protein or PB1, the hlHA, hlHA/M2e and PB 1 genes were chemically synthesized (Geneart, Inc., Regensburg, Germany). The synthetic genes are driven by the strong vaccinia early/late promoter mH5 (Wyatt et al. 1996) and terminated with a vaccinia virus specific stop signal downstream of the coding region that is absent internally. The gene cassettes were cloned in the plasmid pDM-D4R (Ricci et al., 2011) resulting in plasmids pDM-hlHA, pDM-hlHA/M2e and pDM-PB1-VN, respectively. The introduction of the foreign genes into the D4R/D5R intergenic region of MVA was done as described elsewhere (Ricci et al. 2011) resulting in viruses MVA-hlHA, MVA-hlHA/M2e, MVA-PB1-VN.

For the construction of the rMVA expressing M1, the M1 sequence (accession number AY818144) was placed downstream of the strong vaccinia early/late promoter selfP (Chakrabarti et al. 1997) and cloned in pDM-D4R, resulting in pDM-M1-VN. The expression cassette of pDD4-M2-VN—including the M2 sequence (accession number EF541453) under the control of the mH5 promoter—was cloned in pDM-D4R resulting in pDM-M2-VN. The plasmids were then used for recombination with MVA according to Holzer et al, *supra* resulting in the viruses MVA-M1-VN and MVA-M2-VN, respectively as shown in FIG. 5A.

For the construction of single-insert MVAs expressing the NP protein, the NP expression cassette of pDD4-mH5-mNP-VN (Mayrhofer et al., *supra*) was cloned in plasmid pd3-lacZ-gpt, resulting in pd3-lacZ-mH5-NP-VN. Plasmid pd3-lacZ-gpt contains a lacZ/gpt selection marker cassette and a multiple cloning site (MCS) for insertion of genes of interest. The sequences are framed by genomic MVA sequences of the del III region. The marker cassette is destabilized by a tandem repeat of MVA del III flank, thus the final recombinant is free of any auxiliary sequences. The insertion plasmid directs the gene cassettes into the MVA deletion III (del III) region. After infection of primary chicken embryo cells with MOI 1, cells were transfected with pd3-lacZ-mH5-NP-VN according to the calcium phosphate technique (Graham and van der Eb 1973), resulting MVA-NP-VN shown in FIG. 5B. The MVA strain (MVA 1974/NIH clone 1) was kindly provided by B. Moss (National Institutes of Health). Recombinant virus is selected using the transient marker stabilization method (Scheiflinger et al, 1998).

The single-insert MVA vectors expressing the NP, PB1, M1, M2, hIHA, and hIHA/M2e were characterized by PCR and Western blot as described in Hessel et al, *supra*. Recombinant viruses were grown in CEC or DF-1 cells and purified by centrifugation through a sucrose cushion. Primary CEC were produced in-house and cultivated in Med199 (Gibco®) supplemented with 5% fetal calf serum (FCS). The DF-1 (CRL-12203) cell line was obtained from the ATCC (American Type Culture Collection) and cultivated in DMEM (Biochrom, Inc.) supplemented with 5% FCS.

The correct expression of the influenza proteins by the rMVAs was confirmed by Western blotting. For this purpose CEC or the permanent chicken cell line DF-1 were infected with a MOI of 0.1 and cell lysates were prepared 48-72 hrs post infections. The recombinant MVAs that express the hIHA (MVA-hIHA and MVA-hIHA/M2e) were analyzed in a Western blot using an anti-influenza A/Vietnam/1194/04 (H5N1) polyclonal serum (NIBSC 04/214) for detection. Donkey-anti-sheep alkaline phosphatase-conjugated IgG (Sigma Inc.) was used as a secondary antibody. The recombinant MVAs that express the M2 and M2e (MVA-M2-VN and MVA-hIHA/M2e) were analyzed in Western Blots using an anti-avian influenza M2 antibody binding a peptide present at the amino terminus of the H5N1 M2 (ProSci, Cat#4333). Goat-anti-rabbit alkaline phosphatase-conjugated IgG (Sigma Inc.) antibody was used as a secondary antibody. As shown in FIG. 6A, the recombinant MVAs expressing the hIHA (MVA-hIHA and MVA-hIHA/M2e) gene inserts induced expression of the HA containing antigens in avian DF-1 cells. The bands around 40 kDa in lane 2 represent the hIHA. The larger band at around 70 kDa in lane 3 represents the hIHA/M2e. The large band at around 80 kDa in lane 5 represents the HA0 hemagglutinin-precursor, which is cleaved into the HA1 and HA2 subunits represented by the bands at approximately 55 and 25 kDa. The specific hIHA, hIHA/M2e or HA bands are absent in the wild-type MVA control (lane 4).

FIG. 6B shows the M2 expression by MVA-M2-VN (lane 2) or MVA-hIHA/M2e (lane 3). The weak but specific band around 10 kDa in lane 2 represents the wild-type M2 protein

whereas the larger band around 70 kDa represents the hIHA/M2e protein. Both bands are absent in the wild-type MVA control (lane 4).

The expression of the M1, NP and PB1 protein is detected with polyclonal guinea-pig anti-influenza H5N1 serum produced in house, a polyclonal goat antibody detecting the PB1 of Influenza A virus (Santa Cruz, Cat#: vC-19), and a monoclonal mouse-anti-NP-antibody (BioXcell, Cat# BE0159), respectively. The MVA-M1-VN and MVA-NP-VN induce expression of the M1 protein (around 27 kDa) and the NP protein (around 60 kDa) (not shown).

### Example 3

#### Animal Experiments with the Single-Insert Vaccines

##### Protection Experiment

A standard protection experiment consists of two arms (primed with about  $1 \times 10^3$ - $1 \times 10^5$  TCID<sub>50</sub> H1N1v CA/07 and unprimed) of nine groups of mice each (respectively vaccinated i.m. with  $1 \times 10^6$  pfu of the nine vaccines and controls shown in Table 2), a group consisting of six animals resulting in 108 animals, defines one set. The animals of one set are challenged with one of the six challenge viruses shown in Table 3 below.

TABLE 3

Pre-treatment	Challenge strain	Subtype	Abbreviation
H1N1v/unprimed	A/California/07/2009	H1N1	CA/07
H1N1v/unprimed	A/Vietnam/1203/2004	H5N1	VN/1203
H1N1v/unprimed	A/HongKong/G9/	H9N2	HK/G9
H1N1v/unprimed	A/Victoria/210/2009	H3N2	VI/09
H1N1v/unprimed	A/FPV/Rostock/34	H7N1	RO/34
H1N1v/unprimed	A/PR8/1934	H1N1	PR8

Female Balb/c mice are 8-10 weeks old at the pre-treatment time point and 14-16 weeks old at the time point of immunization with the vaccines and controls shown in Table 2. Mice were immunized intramuscularly twice (days 42 and 63) with  $10^6$  pfu of the vaccines or wild type MVA, 3.75 µg whole virus preparation H9N2 A/HongKong/G9/1997 or with buffer (PBS). At day 84, mice were challenged intranasally with  $10^3$  TCID<sub>50</sub> H5N1 A/Vietnam/1203/2004 (H5N1, CDC #2004706280), with  $2.5 \times 10^4$  TCID<sub>50</sub> mouse adapted H9N2 A/HongKong/G9/1997 or with  $1.66 \times 10^4$  TCID<sub>50</sub> H7N1 A/FPV/Rostock/34. The challenge doses correspond to approx. 30 LD50 for the H5N1 challenge and 32 LD50 for the H9N2 challenge per animal. Sera are collected at days 41, 62 and 85 and analyzed for HA-specific IgG concentration by HI titer or microneutralization assay.

The primary outcome of the animal experiments is protection as measured by lethal endpoint, weight loss, or lung titer. Further the ELISA titers of pooled pre-challenge sera measured against inactivated whole virus H5N1 strain A/Vietnam/1203/2004 are determined.

##### T Cell Experiments

Frequencies of influenza-specific CD4 and CD8 T cells are determined in immunized mice by flow cytometry. In a standard experiment, groups of 5 female BALB/c mice are immunized twice with the vaccines or controls listed in Table 2. Splenocytes are re-stimulated in-vitro using inactivated whole virus antigens of different influenza strains for CD4 T-cells and, when available, peptides representing the CD8 T-cell epitopes of the vaccine insert constructs and IFN-γ production are measured. All experiments are performed twice, using a total of 140 animals.

## Other Experiments

An evaluation of the cell-mediated immunity after a single immunization, demonstration of functional activity of cytotoxic T-cells in a VITAL assay and assessment of recruitment of influenza-specific T-cells into the lungs of challenged animals are also carried out. The induction/expansion of vaccine-specific T-cells is also monitored in the primed mouse model by immunizing mice which resolved a influenza virus infection once with these vaccines.

## Example 4

## Construction and Characterization of Double-Insert rMVA Vectors

The following double-insert, rMVA and controls are utilized in the experiments described herein.

TABLE 4

rMVA	Inserted influenza gene(s)	Comment
1. MVA-hlHA-NP	headless HA + NP	Double insert construct
2. MVA-hlHA/M2e-NP	headless HA/m2e fusion protein + NP	Double insert construct
3. MVA-NP-VN	nucleoprotein	Control
4. MVA-HA-VN	hemagglutinin	Control
5. MVA-wt	empty vector	Neg. control
6. PBS	—	Neg. control

For the construction of the double insert rMVA vector co-expressing either the hlHA or hlHA/M2e gene cassette in combination with the NP protein gene cassette, the single insert MVA recombinants of Example 2 containing the hlHA or hlHA/M2e gene cassette are used. CEC cells were infected with MVA-hlHA or MVA-hlHA/Me2 and afterwards transfected with pd3-lacZ-mH5-NP-VN (see Example 2). Homologous recombination and propagation of the recombinant MVA vectors are performed as described in Example 2. The resulting double insert MVA vectors, named MVA-hlHA-NP or MVA-hlHA/M2e-NP, contain the hlHA or hlHA/M2e expression cassette in the D4R/D5R locus and the NP expression cassette in the del III locus. See FIG. 7.

The recombinant MVAs were characterized by Western Blot as described in Example 2. FIG. 8A shows the expression of the hlHA and hlHA/M2e after infection of CEC with MVA-hlHA-NP (lane 6) or MVA-hlHA/M2e-NP (lane 5). The bands around 40 kDa in lanes 3 and 6 represent the hlHA of the MVA-hlHA and MVA-hlHA-NP constructs. The band around 70 kDa in lane 5 represents the hlHA/M2e fusion protein. The HA bands are absent in the wild-type control in lane 4. The same samples were used for detection of NP protein expression in Western Blots (as described in Example 2). As shown in FIG. 8B, the recombinant MVAs MVA-hlHA-NP and MVA-hlHA/M2e-NP also induced expression of the NP protein in avian CEC cells. The bands around 60 kDa in lanes 2 to 4 represent the NP.

## Example 5

## Animal Experiments with the Double-Insert Vaccines or Vector Combinations

## Protection Experiment

A standard experiment included eight groups of mice (vaccinated with the six vaccines and controls shown in Table 5) each group consisting of six animals. The protection experiments were carried out as described in Example

3. After challenge mice were monitored over a time period of 14 days and weight loss or symptoms including ruffled fur (score of 1), curved posture (score of 2), apathy (score of 3), and death (score of 4) were recorded. For ethical reasons, mice were euthanized after weight loss of >25%. Protection results are compiled in Table 5 and displayed in FIGS. 9 and 10.

TABLE 5

Protection of mice from death after double dose vaccinations with recombinant MVAs and homologous or heterologous challenge.					
Gr.	Vaccine	After H5N1 VN1203 <sup>(1)</sup> challenge		After H9N2 HK/G9 <sup>(2)</sup> challenge	
		Clinical score at day 14	Protection n/n <sup>(3)</sup> (%)	Clinical score at day 14	Protection n/n (%)
1	MVA-hlHA-NP	2.83	2/6 (33)	0	6/6 (100)
2	MVA-hlHA/M2e-NP	1	5/6 (83)	0	6/6 (100)
3	MVA-hlHA	2.67	2/6 (33)	3.33	1/6 (17)
4	MVA-hlHA/M2e	4	0/6 (0)	2.67	2/6 (33)
5	MVA-NP-VN	3.33	2/6 (33)	0	6/6 (100)
6	Homologous control vaccine <sup>(4)</sup>	0	6/6 (100)	0	6/6 (100)
7	MVA-wt <sup>(5)</sup>	4	0/6 (0)	2.83	2/6 (33)
8	PBS	2.67	2/6 (33)	4	0/6 (0)

<sup>(1)</sup>VN1203, challenge strain A/Vietnam/1203/2004;

<sup>(2)</sup>HK/G9, challenge strain A/HongKong/G9/1997;

<sup>(3)</sup>n/n, survival per group;

<sup>(4)</sup>Homologous control vaccine;

<sup>(5)</sup>wild-type MVA (NIH74 LVD clone 6).

As positive control mice were vaccinated with homologous control constructs. In case of H5N1 challenge mice 35 were vaccinated with MVA-HA-VN (Hessel et al., 2011) and in case of H9N2 challenge mice were vaccinated with an inactivated whole virus preparation of the H9N2 A/Hong-Kong/G9/1997 influenza virus. Both controls induced full protection (Table 5; FIGS. 9 and 10, panels E). In the 40 wild-type MVA and buffer groups all mice showed marked weight loss compared to the positive control groups and nearly all mice died after challenge. Mice vaccinated with the single recombinant MVAs (MVA-hlHA, MVA-hlHA/M2e, MVA-D3-NP-VN) showed no significantly better protection after the strong H5N1 challenge compared to the negative control groups (FIG. 9 A-F). Also against heterosubtypic (H9N2) challenge no significant protection was seen in MVA-hlHA and MVA-hlHA/M2e vaccinated groups (FIG. 10).

Surprisingly, however, vaccination with the double construct expressing the fusion protein hlHA/M2e and the NP protein resulted in nearly full protection (FIG. 9 B, D, F) after the H5N1 challenge with approx. 30 LD50 per animal. Also after heterosubtypic challenge (with approx. 32 LD50 55 H9N2 virus) mice were fully protected after vaccination with the double recombinant MVA-hlHA/M2e-NP. Furthermore, the double recombinant MVA-hlHA-NP and the single recombinant MVA-NP-VN induced full protection against the heterosubtypic challenge with H9N2 (FIG. 10, B, D, F). As can be seen in the weight monitoring (FIGS. 9 and 10, panels B) and in the clinical scores (FIGS. 9 and 10, panels D), the double construct MVA-hlHA/M2e-NP showed the best results presumably by combined beneficial effects contributed by the different influenza antigens.

## T Cell Experiments

Frequencies of influenza-specific CD4 and CD8 T cells are determined in immunized mice by flow cytometry. In a

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standard protocol experiment, groups of 5 female BALB/c mice are immunized twice with the vaccines or controls listed in Table 4. Splenocytes are re-stimulated in-vitro using inactivated whole virus antigens of different influenza strains for CD4 T-cells and, when available, peptides representing the CD8 T-cell epitopes of the vaccine insert constructs and IFN- $\gamma$  production are measured. All experiments are performed twice.

#### Other Experiments

An evaluation of the cell mediated immunity after a single immunization, demonstration of functional activity of cytotoxic T-cells in a VITAL assay and assessment of recruitment of influenza-specific T-cells into the lungs of challenged animals are also carried out. The induction/expansion of vaccine-specific T-cells is also monitored in the primed mouse model by immunizing mice which resolved a influenza virus infection once with these vaccines.

#### Example 6

##### Construction and Characterization of Triple-Insert rMVA Vectors and Virus-Like Particles

Influenza virus-like particles (VLPs) induce humoral and cellular responses and can protect against lethal challenges (Bright et al. 2007; Pushko et al. 2005; Song et al. 2010). VLPs chosen for experiments herein comprise either hlHA or hlHA/M2e in combination with NP and M1. The VLPs are generated from triple-insert MVA vectors.

For the construction of the triple-insert MVA vectors co-expressing either hlHA or hlHA/M2e in combination with the M1 (SEQ ID NO: 11) and the NP protein (SEQ ID NO: 13), the M1 gene (SEQ ID NO: 10) of pDD4-M1-VN is cloned downstream of the synthetic early/late promotor selP (Chakrabarti et al. 1997). The resulting gene cassette is cloned downstream of the hlHA or hlHA/M2e gene cassette in pDM-hlHA or pDM-hlHA/M2e. The resulting plasmids harboring a double gene cassette (pDM-hlHA-M1 and pDM-hlHA/M2e-M1) are used for recombination into defective MVA as described above. Afterwards, a recombination with an NP gene cassette (SEQ ID NO: 12)-containing plasmid (pD3-lacZ-gpt-NP-VN) is done resulting in a triple-insert MVA virus. This triple-insert MVA is plaque purified under transient marker selection.

The triple-insert MVA vectors, named MVA-hlHA-M1-NP or MVA-hlHA/M2e-M1-NP contain the hlHA or hlHA/M2e expression cassette and M1 expression cassette in tandem order in the D4R/D5R locus and the NP expression cassette in the del III locus (FIG. 7).

Detection of VLPs is as follows. HeLa or 293 cells are seeded into T175 cm<sup>2</sup> flasks and grown in DMEM+10% FCS+Pen/Strep. To generate VLPs, cells are infected with 1 MOI of single-insert MVA or triple-insert MVA recombinants, respectively. Empty MVA vectors or single-insert MVA recombinants without M1 are used as controls. At 1 h post infection (p.i.), the medium is replaced by DMEM+Pen/Strep and culture medium is harvested 48 h p.i. and cellular debris is pelleted by centrifugation at 2.000×g for 10 min. The procedure for analyzing VLPs by sucrose gradient density flotation and sucrose cushion has been described previously (Chen et al. 2007; Chen et al. 2005; Gomez-Puertas et al. 2000). The samples are then analyzed by immunoblotting. Additionally, electron microscopy (EM) analysis with medium of infected cells is performed.

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#### Example 7

##### Animal Experiments with the Triple-Insert Vaccines or Vector Combinations

A standard experiment includes 6 groups of primed and unprimed mice (vaccinated with the 6 vaccines and controls shown in Table 5), each group consisting of 6 animals, resulting in 36 animals (1 set). The animals are challenged with one of the 6 challenge viruses shown in Table 3. In sum, there are 6 sets of 72 animals each requiring 432 mice to assess cross-protection in the primed and naive models.

TABLE 5

rMVA	Inserted influenza gene(s)	comment
1. MVA-hlHA-M1-NP	headless HA + nucleoprotein + matrix 1	3 inserts
2. MVA-hlHA/M2e-M1-NP	headless HA/m2e fusion protein + nucleoprotein + matrix 1	3 inserts
3. MVA-tbd	best construct from previous screening	control
4. MVA-HA-VN	hemagglutinin	control
5. MVA-wt	empty vector	neg. control
6. PBS	—	neg. control

The present invention is illustrated by the foregoing examples and variations thereof will be apparent to those skilled in the art. Therefore, no limitations other than those set out in the following claims should be placed on the invention.

All documents cited in this application are hereby incorporated by reference in their entirety for their disclosure described.

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27

28

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<223> OTHER INFORMATION: M1 sequence

<400> SEQUENCE: 10

atgagtcttc taacccgaggt cgaaacgtac gttctctcta tcataccgtc aggccccctc	60
aaaggccgaga tcgcacagaa acttgaagat gtctttgcag gaaagaacac cgatctcgag	120
gctctcatgg agtggctaaa gacaagagcca atcctgtcac ctctgactaa agggatttg	180
ggatttgtat tcacgctcac cgtgcccagt gagcggggac tgcagegtag acgctttgtc	240
cagaatgccc taaatggaaa tggagatcca aataatatgg atagggcagt taagctatat	300
aagaagctga aaagagaaaat aacattccat ggggctaagg aggtcgcaact cagctactca	360
accgggtcac ttgccagttt catgggtctc atataacaaca ggatggaaac ggtgactacg	420
gaagttggctt ttggccatgt gtgtgccact tggagcaga ttgcagatc acagcatcg	480
tctcacagac agatggcaac tatcaccaac ccactaatca gacatgagaa cagaatgg	540
ctggccagca ctacagctaa ggctatggag cagatggcgg gatcaagtga gcaggcagcg	600
gaagccatgg agatcgctaa tcaggctagg cagatggcgc aggcaatgag gacaattgg	660
actctatccta actcttagtgc tggctctgaga gataatcttc ttgaaaattt gcaggcctac	720
cagaaacgaa tgggagtgca gatgcagcga ttcaagtgtat cctattgtt ttgcgcacaa	780
tatcattggg atcttgcact tggatattgtt gattcttgat cgtctttct tcaaattgtat	840
ttatcgctgc cttaaaatacg gtttggaaag agggcctgct acggcagggg tacctgagtc	900
tatgaggggaa gagtaccggc aggaacagca gagtgcgttg gatgttgacg atggtcattt	960
tgtcaacata gaattggagt aaaaaaa	986

<210> SEQ ID NO 11  
<211> LENGTH: 252  
<212> TYPE: PRT  
<213> ORGANISM: Influenza A virus  
<220> FEATURE:  
<221> NAME/KEY: MISC\_FEATURE  
<223> OTHER INFORMATION: M1 sequence

<400> SEQUENCE: 11

Met Ser Leu Leu Thr Glu Val Glu Thr Tyr Val Leu Ser Ile Ile Pro  
 1               5               10               15

-continued

Ser Gly Pro Leu Lys Ala Glu Ile Ala Gln Lys Leu Glu Asp Val Phe  
20 25 30

Ala Gly Lys Asn Thr Asp Leu Glu Ala Leu Met Glu Trp Leu Lys Thr  
35 40 45

Arg Pro Ile Leu Ser Pro Leu Thr Lys Gly Ile Leu Gly Phe Val Phe  
50 55 60

Thr Leu Thr Val Pro Ser Glu Arg Gly Leu Gln Arg Arg Arg Phe Val  
65 70 75 80

Gln Asn Ala Leu Asn Gly Asn Gly Asp Pro Asn Asn Met Asp Arg Ala  
85 90 95

Val Lys Leu Tyr Lys Lys Leu Lys Arg Glu Ile Thr Phe His Gly Ala  
100 105 110

Lys Glu Val Ala Leu Ser Tyr Ser Thr Gly Ala Leu Ala Ser Cys Met  
115 120 125

Gly Leu Ile Tyr Asn Arg Met Gly Thr Val Thr Glu Val Ala Phe  
130 135 140

Gly Leu Val Cys Ala Thr Cys Glu Gln Ile Ala Asp Ser Gln His Arg  
145 150 155 160

Ser His Arg Gln Met Ala Thr Ile Thr Asn Pro Leu Ile Arg His Glu  
165 170 175

Asn Arg Met Val Leu Ala Ser Thr Thr Ala Lys Ala Met Glu Gln Met  
180 185 190

Ala Gly Ser Ser Glu Gln Ala Ala Glu Ala Met Glu Ile Ala Asn Gln  
195 200 205

Ala Arg Gln Met Val Gln Ala Met Arg Thr Ile Gly Thr His Pro Asn  
210 215 220

Ser Ser Ala Gly Leu Arg Asp Asn Leu Glu Asn Leu Gln Ala Tyr  
225 230 235 240

Gln Lys Arg Met Gly Val Gln Met Gln Arg Phe Lys  
245 250

<210> SEQ ID NO 12  
<211> LENGTH: 1507  
<212> TYPE: DNA  
<213> ORGANISM: Influenza A virus  
<220> FEATURE:  
<221> NAME/KEY: misc\_feature  
<223> OTHER INFORMATION: NP sequence

<400> SEQUENCE: 12

atggcgtctc aaggcaccaa acgatcttat gaacagatgg aaactggtgg ggaacgc	cag 60
aatgctactg agatcagggc atctgttgg aagaatggta gtggcattgg gaggttctac	120
atacagatgt gcacagaact caaactcagt gactatgaag ggaggctgat ccagaacagc	180
ataacaatag agagaatggt actctctgca tttgatgaaa gaaggaacag atacctggaa	240
gaacacccca gtgcgggaaa ggaccgaaag aagactggag gtcccaattta tcggaggaga	300
gacggaaat gggtagaga gctaattctg tacgacaaag aggagatcg gaggatttg	360
cgtcaaggca acaatggaga ggacgcact gctggcttta cccacctgat gatatggcat	420
tccaatctaa atgatgccac atatcagaga acgagagctc tcgtgcgtac tggaatggac	480
ccaaggatgt gctctctgat gcaagggtca actctcccgaa ggagatctgg agctgccgt	540
gcagcgttaa aggggtagg gacaatggtg atggagctga ttccggatgat aaaacgagg	600
atcaacgacc ggaatttctg gagaggcgaa aatggaagaa gaacaaggat tgcatatgag	660
agaatgtgca acatcctcaa agggaaattc caaacagcag cacaaagagc aatgatggat	720

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caagtgcgag agagcagaaaa tcctggaaat gctgaaattt aagatctcat ttttctggca    780
cggctctgcac tcatacctgag aggatcagtg gcccataagt cctgcttgcg tgcttgcgtg    840
tacggacttg cagtggccag tggatatgac tttgagagag aagggtactc tctggttgga    900
atagatcctt tccgcctgtct tcaaaacagc caggtctta gtctcattag accaaatgag    960
aatccagcac ataagagtca attagtgtgg atggcatgcc actctgcagc atttgaggac   1020
cttagagtct caagtttcat cagagggaca agagtggcc caagaggaca gctatccacc   1080
agaggggttc aaattgcttc aaatgagaac atggaggcaa tggactccaa cactttgaa   1140
ctgagaagca gatattgggc tataagaacc agaagcggag gaaacaccaa ccagcagagg   1200
geatctgcag gacagatcag cgttcagccc actttctcggttccagagaaa cttcccttc   1260
gaaagagcga ccattatggc agcatttaca gaaatactg agggcagaac gtctgacatg   1320
aggactgaaa tcataagaat gatggaaagt gccagaccag aagatgtgtc attccagggg   1380
ccccggggactct tcgagctctc ggacggaaag gcaacgaacc cgatcgtgcc ttcccttgac   1440
atgaataatg aaggatctta ttcttcgga gacaatgcag aggagtatga caattaaaga   1500
aaaatac                                              1507

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<210> SEQ_ID NO 13
<211> LENGTH: 498
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: NP sequence

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<400> SEQUENCE: 13
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Met Ala Ser Gln Gly Thr Lys Arg Ser Tyr Glu Gln Met Glu Thr Gly
1           5          10          15

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```

Gly Glu Arg Gln Asn Ala Thr Glu Ile Arg Ala Ser Val Gly Arg Met
20          25          30

```

```

Val Ser Gly Ile Gly Arg Phe Tyr Ile Gln Met Cys Thr Glu Leu Lys
35          40          45

```

```

Leu Ser Asp Tyr Glu Gly Arg Leu Ile Gln Asn Ser Ile Thr Ile Glu
50          55          60

```

```

Arg Met Val Leu Ser Ala Phe Asp Glu Arg Arg Asn Arg Tyr Leu Glu
65          70          75          80

```

```

Glu His Pro Ser Ala Gly Lys Asp Pro Lys Lys Thr Gly Pro Ile
85          90          95

```

```

Tyr Arg Arg Asp Gly Lys Trp Val Arg Glu Leu Ile Leu Tyr Asp
100         105         110

```

```

Lys Glu Glu Ile Arg Arg Ile Trp Arg Gln Ala Asn Asn Gly Glu Asp
115         120         125

```

```

Ala Thr Ala Gly Leu Thr His Leu Met Ile Trp His Ser Asn Leu Asn
130         135         140

```

```

Asp Ala Thr Tyr Gln Arg Thr Arg Ala Leu Val Arg Thr Gly Met Asp
145         150         155         160

```

```

Pro Arg Met Cys Ser Leu Met Gln Gly Ser Thr Leu Pro Arg Arg Ser
165         170         175

```

```

Gly Ala Ala Gly Ala Ala Val Lys Gly Val Gly Thr Met Val Met Glu
180         185         190

```

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Leu Ile Arg Met Ile Lys Arg Gly Ile Asn Asp Arg Asn Phe Trp Arg
195         200         205

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Gly Glu Asn Gly Arg Arg Thr Arg Ile Ala Tyr Glu Arg Met Cys Asn  
210 215 220

Ile Leu Lys Gly Lys Phe Gln Thr Ala Ala Gln Arg Ala Met Met Asp  
225 230 235 240

Gln Val Arg Glu Ser Arg Asn Pro Gly Asn Ala Glu Ile Glu Asp Leu  
245 250 255

Ile Phe Leu Ala Arg Ser Ala Leu Ile Leu Arg Gly Ser Val Ala His  
260 265 270

Lys Ser Cys Leu Pro Ala Cys Val Tyr Gly Leu Ala Val Ala Ser Gly  
275 280 285

Tyr Asp Phe Glu Arg Glu Gly Tyr Ser Leu Val Gly Ile Asp Pro Phe  
290 295 300

Arg Leu Leu Gln Asn Ser Gln Val Phe Ser Leu Ile Arg Pro Asn Glu  
305 310 315 320

Asn Pro Ala His Lys Ser Gln Leu Val Trp Met Ala Cys His Ser Ala  
325 330 335

Ala Phe Glu Asp Leu Arg Val Ser Ser Phe Ile Arg Gly Thr Arg Val  
340 345 350

Val Pro Arg Gly Gln Leu Ser Thr Arg Gly Val Gln Ile Ala Ser Asn  
355 360 365

Glu Asn Met Glu Ala Met Asp Ser Asn Thr Leu Glu Leu Arg Ser Arg  
370 375 380

Tyr Trp Ala Ile Arg Thr Arg Ser Gly Gly Asn Thr Asn Gln Gln Arg  
385 390 395 400

Ala Ser Ala Gly Gln Ile Ser Val Gln Pro Thr Phe Ser Val Gln Arg  
405 410 415

Asn Leu Pro Phe Glu Arg Ala Thr Ile Met Ala Ala Phe Thr Gly Asn  
420 425 430

Thr Glu Gly Arg Thr Ser Asp Met Arg Thr Glu Ile Ile Arg Met Met  
435 440 445

Glu Ser Ala Arg Pro Glu Asp Val Ser Phe Gln Gly Arg Gly Val Phe  
450 455 460

Glu Leu Ser Asp Glu Lys Ala Thr Asn Pro Ile Val Pro Ser Phe Asp  
465 470 475 480

Met Asn Asn Glu Gly Ser Tyr Phe Phe Gly Asp Asn Ala Glu Glu Tyr  
485 490 495

Asp Asn

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<210> SEQ ID NO 14
<211> LENGTH: 1026
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic nucleotide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: h1HA sequence
<220> FEATURE:
<221> NAME/KEY: misc_signal
<222> LOCATION: (1)..(48)
<223> OTHER INFORMATION: Signal sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (49)..(174)
<223> OTHER INFORMATION: HAI nucleotides
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (175)..(186)
<223> OTHER INFORMATION: Linker
<220> FEATURE:

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<221> NAME/KEY: misc_feature
<222> LOCATION: (187)..(348)
<223> OTHER INFORMATION: HA1 nucleotides
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (349)..(1026)
<223> OTHER INFORMATION: HA2 stalk region

<400> SEQUENCE: 14

atggagaaaa tagtgcttct ttttgcataa gtcagtcgtt ttaaaagtga tcagattgc      60
atgggttacc atgcaaacaa ctcgacagag cagggtgaca caataatgga aaagaacgtt      120
actgttacac atgcccaga acaactggaa aagaaacaca acgggaagct ctgcggagga      180
ggaggatgca acaccaagtg tcaaactcca atggggcga taaactctag catgccattc      240
cacaatatac accctctcac cattggggaa tgcccaaat atgtgaaatc aaacagatta      300
gtccttgcga ctgggctcag aaatagccct caaagagaga gaagaagaaa aaagagagga      360
ttatggtag ctatagcagg tttttagag ggaggatggc agggaatggt agatggtagg      420
tatgggttacc accatagcaa tgagcagggg agtgggtacg ctgcagacaa agaatccact      480
caaaaggcaa tagatggagt caccaataag gtcaactcga tcattgacaa aatgaacact      540
cagtttgagg ccgttggaaag ggaatttaac aacttagaaa ggagaataga gaatttaaac      600
aagaagatgg aagacgggtt cctagatgtc tggacttata atgctgaact tctgggtctc      660
atggaaaatg agagaactct agactttcat gactcaaatg tcaagaacct ttacgacaag      720
gtccgactac agcttaggga taatgc当地 gagctggta acgggtgtt cgagttctat      780
cataaatgtg ataatgaatg tatggaaatg gtaagaaatg gaacgtatga ctaccccgag      840
tattcagaag aagcgagact aaaaagagag gaaataatgtg gagtaaaattt ggaatcaata      900
ggaatttacc aaatactgtc aatttattct acagtggcga gttcccttagc actggcaatc      960
atggtagctg gtctatcctt atggatgtgc tccaaatggat cgttacaatg cagaatttgc     1020
atttaa                                         1026

<210> SEQ_ID NO 15
<211> LENGTH: 341
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: h1HA sequence
<220> FEATURE:
<221> NAME/KEY: SIGNAL
<222> LOCATION: (1)..(16)
<223> OTHER INFORMATION: Signal sequence
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (17)..(58)
<223> OTHER INFORMATION: HA1 residues
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (59)..(62)
<223> OTHER INFORMATION: Linker
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (63)..(116)
<223> OTHER INFORMATION: HA1 residues
<220> FEATURE:
<221> NAME/KEY: SITE
<222> LOCATION: (112)..(119)
<223> OTHER INFORMATION: Polybasic cleavage site
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (117)..(341)

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&lt;223&gt; OTHER INFORMATION: HA2 residues

&lt;400&gt; SEQUENCE: 15

Met	Glu	Lys	Ile	Val	Leu	Leu	Phe	Ala	Ile	Val	Ser	Leu	Val	Lys	Ser
1					5				10				15		

Asp	Gln	Ile	Cys	Ile	Gly	Tyr	His	Ala	Asn	Asn	Ser	Thr	Glu	Gln	Val
					20				25				30		

Asp	Thr	Ile	Met	Glu	Lys	Asn	Val	Thr	Val	Thr	His	Ala	Gln	Asp	Ile
						35		40		45					

Leu	Glu	Lys	Lys	His	Asn	Gly	Lys	Leu	Cys	Gly	Gly	Gly	Cys	Asn
					50		55		60					

Thr	Lys	Cys	Gln	Thr	Pro	Met	Gly	Ala	Ile	Asn	Ser	Ser	Met	Pro	Phe
					65		70		75				80		

His	Asn	Ile	His	Pro	Leu	Thr	Ile	Gly	Glu	Cys	Pro	Lys	Tyr	Val	Lys
					85		90		95						

Ser	Asn	Arg	Leu	Val	Leu	Ala	Thr	Gly	Leu	Arg	Asn	Ser	Pro	Gln	Arg
					100			105		110					

Glu	Arg	Arg	Lys	Lys	Arg	Gly	Leu	Phe	Gly	Ala	Ile	Ala	Gly	Phe
					115		120		125					

Ile	Glu	Gly	Gly	Trp	Gln	Gly	Met	Val	Asp	Gly	Trp	Tyr	Gly	Tyr	His
					130		135		140						

His	Ser	Asn	Glu	Gln	Gly	Ser	Gly	Tyr	Ala	Ala	Asp	Lys	Glu	Ser	Thr
					145		150		155		160				

Gln	Lys	Ala	Ile	Asp	Gly	Val	Thr	Asn	Lys	Val	Asn	Ser	Ile	Ile	Asp
					165			170		175					

Lys	Met	Asn	Thr	Gln	Phe	Glu	Ala	Val	Gly	Arg	Glu	Phe	Asn	Asn	Leu
					180			185		190					

Glu	Arg	Arg	Ile	Glu	Asn	Leu	Asn	Lys	Lys	Met	Glu	Asp	Gly	Phe	Leu
					195		200		205						

Asp	Val	Trp	Thr	Tyr	Asn	Ala	Glu	Leu	Leu	Val	Leu	Met	Glu	Asn	Glu
					210		215		220						

Arg	Thr	Leu	Asp	Phe	His	Asp	Ser	Asn	Val	Lys	Asn	Leu	Tyr	Asp	Lys
					225		230		235		240				

Val	Arg	Leu	Gln	Leu	Arg	Asp	Asn	Ala	Lys	Glu	Leu	Gly	Asn	Gly	Cys
					245		250		255						

Phe	Glu	Phe	Tyr	His	Lys	Cys	Asp	Asn	Glu	Cys	Met	Glu	Ser	Val	Arg
					260		265		270						

Asn	Gly	Thr	Tyr	Asp	Tyr	Pro	Gln	Tyr	Ser	Glu	Glu	Ala	Arg	Leu	Lys
					275		280		285						

Arg	Glu	Glu	Ile	Ser	Gly	Val	Lys	Leu	Glu	Ser	Ile	Gly	Ile	Tyr	Gln
					290		295		300						

Ile	Leu	Ser	Ile	Tyr	Ser	Thr	Val	Ala	Ser	Ser	Leu	Ala	Leu	Ile	Ile
					305		310		315		320				

Met	Val	Ala	Gly	Leu	Ser	Leu	Trp	Met	Cys	Ser	Asn	Gly	Ser	Leu	Gln
					325		330		335						

Cys	Arg	Ile	Cys	Ile											
					340										

&lt;210&gt; SEQ ID NO 16

&lt;211&gt; LENGTH: 2274

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Influenza A virus

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: misc\_feature

&lt;223&gt; OTHER INFORMATION: PB1 sequence

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<400> SEQUENCE: 16

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<210> SEQ\_ID NO 17  
<211> LENGTH: 757  
<212> TYPE: PRT  
<213> ORGANISM: Influenza A virus  
<220> FEATURE:  
<221> NAME/KEY: MISC\_FEATURE  
<223> OTHER INFORMATION: PB1 sequence

<400> SEQUENCE: 17

Met	Asp	Val	Asn	Pro	Thr	Leu	Leu	Phe	Leu	Lys	Val	Pro	Val	Gln	Asn
1						5			10			15			
Ala	Ile	Ser	Thr	Thr	Phe	Pro	Tyr	Thr	Gly	Asp	Pro	Pro	Tyr	Ser	His
			20				25			30					
Gly	Thr	Gly	Thr	Gly	Tyr	Thr	Met	Asp	Thr	Val	Asn	Arg	Thr	His	Gln
	35				40				45						
Tyr	Ser	Glu	Lys	Gly	Lys	Trp	Thr	Thr	Asn	Thr	Glu	Thr	Gly	Ala	Pro
	50					55			60						
Gln	Leu	Asn	Pro	Ile	Asp	Gly	Pro	Leu	Pro	Glu	Asp	Asn	Glu	Pro	Ser
65				70				75			80				
Gly	Tyr	Ala	Gln	Thr	Asp	Cys	Val	Leu	Glu	Ala	Met	Ala	Phe	Leu	Glu
	85					90			95						
Glu	Ser	His	Pro	Gly	Ile	Phe	Glu	Asn	Ser	Cys	Leu	Glu	Thr	Met	Glu
	100					105			110						
Ile	Val	Gln	Gln	Thr	Arg	Val	Asp	Lys	Leu	Thr	Gln	Gly	Arg	Gln	Thr
	115				120				125						
Tyr	Asp	Trp	Thr	Leu	Asn	Arg	Asn	Gln	Pro	Ala	Ala	Thr	Ala	Leu	Ala
	130				135			140							
Asn	Thr	Ile	Glu	Ile	Phe	Arg	Ser	Asn	Gly	Leu	Thr	Ala	Asn	Glu	Ser
145				150				155			160				
Gly	Arg	Leu	Ile	Asp	Phe	Leu	Lys	Asp	Val	Met	Glu	Ser	Met	Asp	Lys
	165				170			175							
Glu	Glu	Met	Glu	Ile	Thr	Thr	His	Phe	Gln	Arg	Lys	Arg	Arg	Val	Arg
	180				185				190						
Asp	Asn	Met	Thr	Lys	Lys	Met	Val	Thr	Gln	Arg	Thr	Ile	Gly	Lys	Lys
	195					200			205						
Lys	Gln	Arg	Leu	Asn	Lys	Lys	Ser	Tyr	Leu	Ile	Arg	Ala	Leu	Thr	Leu
210					215				220						
Asn	Thr	Met	Thr	Lys	Asp	Ala	Glu	Arg	Gly	Lys	Leu	Lys	Arg	Arg	Ala
225					230			235			240				
Ile	Ala	Thr	Pro	Gly	Met	Gln	Ile	Arg	Gly	Phe	Val	Tyr	Phe	Val	Glu
	245				250			255			255				
Thr	Leu	Ala	Arg	Ser	Ile	Cys	Glu	Lys	Leu	Glu	Gln	Ser	Gly	Leu	Pro
	260				265			270							
Val	Gly	Gly	Asn	Glu	Lys	Lys	Ala	Lys	Leu	Ala	Asn	Val	Val	Arg	Lys
	275				280			285							
Met	Met	Thr	Asn	Ser	Gln	Asp	Thr	Glu	Leu	Ser	Phe	Thr	Ile	Thr	Gly
	290				295			300							
Asp	Asn	Thr	Lys	Trp	Asn	Glu	Asn	Gln	Asn	Pro	Arg	Met	Phe	Leu	Ala
305					310			315			320				
Met	Ile	Thr	Tyr	Ile	Thr	Arg	Asn	Gln	Pro	Glu	Trp	Phe	Arg	Asn	Val
	325				330			335			335				
Leu	Ser	Ile	Ala	Pro	Ile	Met	Phe	Ser	Asn	Lys	Met	Ala	Arg	Leu	Gly
	340				345			350							
Lys	Gly	Tyr	Met	Phe	Glu	Ser	Lys	Ser	Met	Lys	Leu	Arg	Thr	Gln	Ile
	355				360			365			365				

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**49****50**

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Pro Ala Glu Met Leu Ala Asn Ile Asp Leu Lys Tyr Phe Asn Glu Leu  
 370 375 380  
 Thr Lys Lys Lys Ile Glu Lys Ile Arg Pro Leu Leu Ile Asp Gly Thr  
 385 390 395 400  
 Ala Ser Leu Ser Pro Gly Met Met Met Gly Met Phe Asn Met Leu Ser  
 405 410 415  
 Thr Val Leu Gly Val Ser Ile Leu Asn Leu Gly Gln Lys Arg Tyr Thr  
 420 425 430  
 Lys Thr Thr Tyr Trp Trp Asp Gly Leu Gln Ser Ser Asp Asp Phe Ala  
 435 440 445  
 Leu Ile Val Asn Ala Pro Asn His Glu Gly Ile Gln Ala Gly Val Asp  
 450 455 460  
 Arg Phe Tyr Arg Thr Cys Lys Leu Val Gly Ile Asn Met Ser Lys Lys  
 465 470 475 480  
 Lys Ser Tyr Ile Asn Arg Thr Gly Thr Phe Glu Phe Thr Ser Phe Phe  
 485 490 495  
 Tyr Arg Tyr Gly Phe Val Ala Asn Phe Ser Met Glu Leu Pro Ser Phe  
 500 505 510  
 Gly Val Ser Gly Ile Asn Glu Ser Ala Asp Met Ser Ile Gly Val Thr  
 515 520 525  
 Val Ile Lys Asn Asn Met Ile Asn Asn Asp Leu Gly Pro Ala Thr Ala  
 530 535 540  
 Gln Met Ala Leu Gln Leu Phe Ile Lys Asp Tyr Arg Tyr Thr Tyr Arg  
 545 550 555 560  
 Cys His Arg Gly Asp Thr Gln Ile Gln Thr Arg Arg Ser Phe Glu Leu  
 565 570 575  
 Lys Lys Leu Trp Glu Gln Thr Arg Ser Lys Ala Gly Leu Leu Val Ser  
 580 585 590  
 Asp Gly Gly Pro Asn Leu Tyr Asn Ile Arg Asn Leu His Ile Pro Glu  
 595 600 605  
 Val Cys Leu Lys Trp Glu Leu Met Asp Glu Asp Tyr Gln Gly Arg Leu  
 610 615 620  
 Cys Asn Pro Leu Asn Pro Phe Val Ser His Lys Glu Ile Glu Ser Val  
 625 630 635 640  
 Asn Asn Ala Val Val Met Pro Ala His Gly Pro Ala Lys Ser Met Glu  
 645 650 655  
 Tyr Asp Ala Val Ala Thr Thr His Ser Trp Ile Pro Lys Arg Asn Arg  
 660 665 670  
 Ser Ile Leu Asn Thr Ser Gln Arg Gly Ile Leu Glu Asp Glu Gln Met  
 675 680 685  
 Tyr Gln Lys Cys Cys Asn Leu Phe Glu Lys Phe Phe Pro Ser Ser Ser  
 690 695 700  
 Tyr Arg Arg Pro Val Gly Ile Ser Ser Met Val Glu Ala Met Val Ser  
 705 710 715 720  
 Arg Ala Arg Ile Asp Ala Arg Ile Asp Phe Glu Ser Gly Arg Ile Lys  
 725 730 735  
 Lys Glu Glu Phe Ala Glu Ile Met Lys Ile Cys Ser Thr Ile Glu Glu  
 740 745 750  
 Leu Arg Arg Gln Lys  
 755

<210> SEQ ID NO 18  
 <211> LENGTH: 99  
 <212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: mH5 promoter

<400> SEQUENCE: 18
aaaaattgaa aataaataca aaggttcttg agggttgtgt taaattgaaa gcgagaaata      60
atcataaata attcattat cgcgatatcc gttaagttt                         99

<210> SEQ ID NO 19
<211> LENGTH: 39
<212> TYPE: DNA
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We claim:

1. A recombinant modified vaccinia virus Ankara (rMVA) comprising a first gene cassette encoding an influenza A headless hemagglutinin (hlHA) polypeptide of SEQ ID NO: 30 15 and a second gene cassette encoding influenza A nucleoprotein (NP).

2. A pharmaceutical composition comprising the rMVA of claim 1.

3. A method of inducing a heterosubtypic immune response to influenza A viruses in an individual comprising administering a pharmaceutical composition comprising the rMVA of claim 1 to the individual.

\* \* \* \* \*